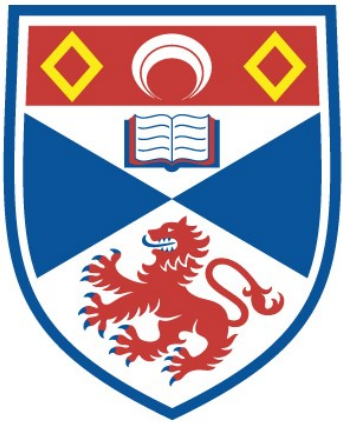
AN ELECTROPHYSIOLOGICAL INVESTIGATION OF THE ACTIONS OF FMRF AMIDE AND RELATED PEPTIDES ON NEURONES OF 'HELIX ASPERSA'

Noel Wyn Davies

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1986

AN ELECTROPHYSIOLOGICAL INVESTIGATION OF THE ACTIONS OF FMRFamide AND RELATED PEPTIDES ON NEURONES OF *HELIX ASPERSA*.

BY NOEL WYN DAVIES

DECLARATIONS

I Noel Wyn Davies hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfillment of any other degree or profesional qualification.

Signed:

Date: 17-12-85

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 on 5:1:82 and as a candidate for the degree of Ph.D. on 5:1:82.

Signed:

Date: 17-12-85

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signature of Supervisor:

Date: 17:12:85

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

ACKNOWLEDGEMENTS

I would like to thank Professor Glen Cottrell for his help and guidence during this research. I am also grateful to Mr. Brian Powell for his general assistance in the laboratory. I very much appreciated the unfailing support that my wife, Margaret, gave me during this endeavour.

I thank the MRC and the SERC for financial support.

ABBREVIATIONS

ABRM	anterior byssus retractor muscle
ACh	acetylcholine
ADH	antidiuretic hormone
4-AP	4-aminopyridine
CCK	cholecystokinin
E_K	potassium equilibrium potential
E_{Na}	sodium equilibrium potential
EPSP	excitatory postsynaptic potential
γ	single channel conductance
GABA	γ-amino butyric acid
gCa	calcium conductance
gCl	chloride conductance
g_{in}	instantaneous conductance
gK	potassium conductance
gNa	sodium conductance
g_{ss}	steady state conductance
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
I	current
Iin	instantaneous current
IPSP	inhibitory postsynaptic potential
Iss	steady state current
Kd	dissociation constant
LHRH	luteinizing hormone releasing hormone
p	rate of channel opening
q	rate of channel closing

TEA	tetraethylammonium
TRH	thyrotropin releasing hormone
V	potential
Vh	holding potential
VIP	vasoactive intestinal peptide

Units:

M	molar
mM	millimolar
ms	millisecond
mV	millivolts
μm	micrometers
MΩ	megaohm
nA	nanoamperes
s	seconds
S	siemens

Amino acid abbreviations:

D	Asp
F	Phe
G	Gly
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
pQ	pyro-Glu

Q	Gln
R	Arg
S	Ser
W	Trp
Y	Tyr

SUMMARY.

1. The actions of FMRFamide and related peptides on identified and some unidentified Helix aspersa neurones have been investigated using electrophysiological techniques.

2. Neurones were voltage clamped using a single microelectrode. The electrode was rapidly switched between recording the membrane potential and passing current using a sample and hold amplifier.

3. Peptides were applied to the neurones by ionophoresis, pressure ejection, or bath application. No qualitative differences were observed between responses induced by different application methods.

4. The ionic mechanisms of the responses were investigated by ion substitution experiments and application of channel blocking compounds.

5. Three responses were induced by FMRFamide: a hyperpolarizing response resulting from a slow increase in gK, a rapidly desensitizing depolarizing response resulting mainly from an increase in gNa, and a voltage dependent response resulting from a suppression of an outward K current (a decrease in gK).

6. Combinations of the above responses occurred in individual neurones. The increase in gK and the increase in gNa occurred simultaneously in the F2 neurone, while the increase in gK and the decrease in gK occurred simultaneously in the C1 neurone.

7. The responses induced by FLRFamide and FIRFamide had similar properties as FMRFamide induced responses. It is concluded that these three tetrapeptides activate the same receptors.

8. Responses to pQDPFLRFamide and YGGFMRFamide were different to those induced by the tetrapeptides. Both of these heptapeptides induced a fast hyperpolarization which was the result of a fast increase in gK, however, they failed to activate the increase in gNa.

9. Four responses were observed to peptides with the common sequence -Met (or Leu) -Arg-Phe-NH_2: a slow increase in gK and an increase in gNa which were activated mainly by the tetrapeptides, and a fast increase in gK and a decrease in gK which were activated mainly by the heptapeptides.

10. The results are consistent with the presence of four types of FMRFamide receptors on Helix neurones.

11. Both FMRFamide and pQDPFLRFamide are present in *Helix*. The different actions of these two peptides reported here imply that they have different physiological roles in *Helix*.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 PEPTIDES AND INTERCELLULAR COMMUNICATION.

Communication between different cells or multicellular structures occurs in most cases by the release of a chemical substance which acts on the target cells. In the endocrine system of multicellular animals, the site of release is often far from the target tissue, and the released substance may linger in the blood or haemolymph of the animal for several hours. In the mammalian endocrine system, many of these intercellular messengers are peptides which may have long-lasting effects on the target tissue.

Communication between neurones, however, requires a brief and localized release of the interneuronal messenger (or neurotransmitter). This requirement is to fulfil the role of the nervous system as a rapid communication system, to integrate and control the functions of multicellular organisms. Compounds which have an established role as neurotransmitters include acetylcholine (ACh), noradrenaline, 5-hydroxytryptamine (5-HT), γ-amino butyric acid (GABA), glutamate and glycine. These compounds are synthesised in the nerve terminal and have been shown to be rapidly degraded by enzymes and/or transported back into the presynaptic nerve terminal following release. A number of monoamines and amino acids have been shown to have neurotransmitter roles throughout the animal kingdom.

In recent years, many peptides have been isolated and characterised from nerve tissue throughout the animal kingdom, and in particular from mammals. These peptides are termed neuropeptides. A wealth of evidence is accumulating which suggests that these neuropeptides have neurotransmitter roles. Neuropeptides, in contrast to monoamines and amino acids, are synthesised in the soma only, and there does not appear to be a reuptake mechanism for released neuropeptides (Hokfelt, Johansson, Ljungdahl, Lundberg and Schultzberg, 1980).

Many compounds have been shown to have multiple actions on neurones, some actions may be associated with a neurotransmitter role while other actions appear to be modulatory. Thus the classification of a substance as a neurotransmitter or neuromodulator can be misleading, as many substances, peptides or non-peptides, may have more than one physiological role. In this chapter, some of the evidence which has accumulated regarding the role of neuropeptides as interneuronal messengers is presented. Since most of the results on neuropeptides has arisen from studies on vertebrate nervous systems, these neuropeptide systems will be discussed first.

1.2 NEUROPEPTIDES IN VERTEBRATES.

1.2.1 LOCALIZATION AND CHARACTERIZATION OF NEUROPEPTIDES.

Biologically active peptides are amongst the most difficult compounds to identify and purify. Some of the major problems arise because of the very small amount of peptide that may be present in the tissue of origin. Nevertheless, a large number of biologically active peptides have been isolated from mammalian nervous tissue and subsequently characterized (Bloom, 1983; Iversen, 1983a,b). Some of the peptides shown to be present in vertebrate neurones which are mentioned in this chapter are: antidiuretic hormone (ADH), cholecystokinin (CCK), luteinizing hormone releasing hormone (LHRH), thyrotropin releasing hormone (TRH), vasoactive intestinal polypeptide (VIP), dynorphin, enkephalin, neurotensin, somatostatin and substance P.

Antibodies have been raised against many of these peptides, thus enabling immunohistochemical detection of peptidergic neurones and processes. These techniques have yielded information on the distribution of various peptide containing neurones and nerve terminals throughout the mammalian nervous system (Hokfelt, Lundberg, Schultzberg, Johansson, Skirboll, Anggard, Fredholm, Hamberger, Pernow, Rehfeld and Goldstein, 1980). Care must be taken in the

interpretation of such data, however, as an antiserum raised against a particular peptide is unlikely to react specifically with that peptide. A positive reaction to an antiserum does not necessarily reflect the presence of a particular peptide within a neuronal system.

1.2.2 LIGAND BINDING STUDIES.

Many peptides can be radiolabelled with isotopes, for example both CCK and VIP may be labelled with ^{135}I, and many others have been tritiated. Such radiolabelled ligands have been used to locate specific binding sites for the ligand in various tissues. Specific binding sites have been located for many neuropeptides in mammalian CNS (Iversen, 1983a). Generally, the localization of binding sites for a particular peptide, coincide with the distribution of nerve processes which are immunoreactive for that peptide.

Using a combination of radioligand binding and immunohistochemical localization of enkephalin, the distributions of opiate binding sites and enkephalin immunoreactive nerve terminals have been found to coincide closely, and to involve central nervous system (CNS) areas which are linked with opiate actions (Snyder, 1980). One of the most striking features of this system is the existence of multiple opioid receptors together with many endogenous ligands for these receptors (Lord, Waterfield,

Hughes and Kosterlitz, 1977; Goldstein and James, 1984; Kosterlitz, 1985). Multiple receptors have also been found for substance P, and the different binding constants for CCK receptors in brain and pancreas suggest the existence of multiple receptors for CCK (Iversen, 1983a). Although much information has been gained from binding studies on the relative selectivity of opioid ligands for the different receptor types, the pharmacological activity of a ligand is determined by the relative numbers of each receptor type present in a given tissue, and also by the efficacy of the ligand at each receptor type. Binding studies merely reflect the association of the ligand and its binding site, which is not necessarily the physiological receptor, and as such cannot distinguish between agonists and antagonists.

1.2.3 RELEASE OF NEUROPEPTIDES.

Several neuropeptides have been shown to be released in a Ca dependent manner following depolarization. Both Met- and Leu-enkephalin are released from synaptosomes produced from corpus striatum. This tissue contains very high endogenous levels of enkephalin. A small basal release of enkephalin occurred from these synaptosomes. This basal release was greatly enhanced following high K or veratridine induced depolarization of the synaptosomes (Henderson, Hughes and Kosterlitz, 1978). Depleting the

concentration of Ca markedly reduced this release. The enkephalin was assayed using the mouse vas deferens as a bioassay system. The depolarization evoked release of enkephalin from slices of rat globus pallidus has also been shown to be Ca dependent (Iversen, Iversen, Bloom, Vargo and Guillemin, 1978). A radioimmunoassay based on an antiserum raised against Leu-enkephalin was used to detect the release.

Other peptides which are released in a Ca dependent manner from various CNS preparations include somatostatin and neurotensin (Iversen, Iversen, Bloom, Douglas, Brown and Vale, 1978; Dodd and Kelly, 1978), substance P (Iversen, Jessell and Kanazawa, 1976; Otsuka and Konishi, 1976), CCK and VIP (Iversen, Lee, Gilbert, Hunt and Emson, 1980).

1.2.4 THE EFFECTS OF NEUROPEPTIDES ON VERTEBRATE NEURONES.

Many of the discovered neuropeptides have an action on both central and peripheral neurones of vertebrates. Much of the investigations performed on the actions of neuropeptides in vertebrates have been made on mammalian CNS using extracellular recording techniques (Renaud and Padjen, 1978). These techniques, while providing valuable information about the effect of a substance on a neuronal system, offer little insight into the cellular mechanism of action. The optimum preparation to study neuronal

actions is one where the affected neurones can be penetrated with an intracellular microelectrode, and where the receptors mediating the response are situated close to the recording site. The examples of neuropeptide actions described here will be limited to those cases where intracellular recording methods were employed.

Application of LHRH onto type B and C sympathetic ganglion neurones of the bullfrog produces a slow depolarization which is accompanied by a decrease in conductance. Analogues of LHRH also produce this response. A voltage clamp investigation of this response revealed a time and voltage dependent K current (the M-current) which is suppressed by LHRH and also muscarinic agonists (Adams and Brown, 1980). At membrane potentials more negative than -60 mV, the response to LHRH, observed as an inward current, was very small, however, at depolarized holding potentials, the response was markedly increased. The properties of the M-current have been analysed in detail (Adams, Brown and Constanti, 1982) and can account for the voltage dependency of the LHRH response. The modulation of this current is proposed to be a mechanism for increasing the excitability of bullfrog ganglion cells by facilitating excitatory postsynaptic potentials (EPSP) (Brown, 1983). In addition to an inhibition of the M-current, LHRH has another depolarizing action in bullfrog ganglion neurones. This response results from an increase in conductance mainly to Na^+

ions. Occasionally, both types of depolarizing responses to LHRH were observed in the same neurone (Katayama and Nishi, 1982).

The M-current is also reduced in a similar manner by substance P (Adams, Brown and Jones, 1983). The responses induced by LHRH and substance P do not occur through activation of the same receptor. Unlike the LHRH response, the substance P induced depolarization in these neurones is desensitized by repeated application, and no interaction is observed between the two peptides (Jan and Jan, 1982). Thus a number of agonists are capable of influencing the M-current in bullfrog sympathetic ganglion neurones.

Substance P depolarizes other neurones in addition to sympathetic ganglion cells. Application of substance P to frog spinal motoneurones induces a slow depolarization which is accompanied by an increase in conductance (Nicoll, 1978). This response, which was also induced by TRH, persisted in the presence of tetrodotoxin. This indicates that substance P and TRH act directly on the recorded neurone. Ionophoretic application of substance P depolarizes cultured mouse spinal neurones (Vincent and Barker, 1979). The depolarization is brief, the decay time being around 100 ms. Sustained application leads to desensitization which recovers rapidly. The response was accompanied by an increase in conductance, and appeared to

reverse close to the peak of the action potential overshoot. Rat spinal dorsal horn neurones are also depolarized by substance P, this effect probably arising from an increase in Na conductance (gNa) and/or Ca conductance (gCa) (Murase and Randic, 1984). In these neurones, substance P was found to influence the Ca spike, although this effect was rather unpredictable. The most consistent effect on the Ca spike was a dose dependent decrease in duration, however, the effect appeared to rely, in an unspecified manner, on the membrane potential prior to spike initiation (Murase and Randic, 1984).

The depolarization elicited by substance P in neurones of the myenteric plexus of the guinea-pig ileum differs from that in the spinal cord because it results from a decrease in conductance (Katayama, North and Williams, 1979). The response was about 1000 times slower than the ACh induced depolarization in the same neurones. It was decreased with hyperpolarization and was sensitive to external K concentration. The authors concluded that the response was a decrease in a K conductance (gK). In a later study on guinea-pig inferior myenteric ganglion neurones, the depolarization induced by substance P was found to consist of two components: a decrease in gK, as described above, and an increase in gNa (Dun and Minota, 1981). These characteristics are similar to those of the non-cholinergic potential recorded in myenteric neurones.

In a voltage clamp study of rat locus coeruleus neurones, North and Williams (1985) described an opioid induced hyperpolarization. This hyperpolarization arises from activation of μ-receptors, which produced an increase in gK. This K current, induced by μ-receptor agonists, was virtually blocked by quinine, and reduced by tetraethylammonium (TEA) and Ba^{2+} ions (North and Williams, 1985).

Another feature of opioid peptides is their ability to reduce the Ca current of sensory neurones. Dunlap and Fischbach (1981) reported that the Ca current, which is activated by depolarization in chick sensory neurones, is decreased by γ-amino butyric acid (GABA), 5-hydroxytryptamine (5-HT), noradrenaline, enkephalin and somatostatin. This effect is believed to be the mechanism by which enkephalin inhibits the release of substance P from these neurones (Mudge, Leeman and Fischbach, 1979).

The somatic action potential of mouse dorsal root ganglion neurones is affected by enkephalin in a similar way to that of chick sensory neurones. Enkephalin decreases the Ca component of the action potential by interacting with either μ- or δ-receptors (Werz and Macdonald, 1982; 1983). Using the agonists morphiceptin and leu-enkephalin and the antagonist naloxone, these authors concluded that there is a variable receptor

distribution amongst dorsal root ganglion neurones. Some neurones express μ-receptors, others express δ-receptors, while some neurones have both types of receptors on their somata. Recently the endogenous opioid peptide dynorphin has been shown to decrease gCa in these neurones (Werz and Macdonald, 1984).

Intracellular recordings of the actions of several other peptides have been accomplished, for example somatostatin and ADH. Somatostatin depolarizes CA1 and CA2 cell bodies in the hippocampus. This excitation is similar to that observed with glutamate, however, no change in membrane conductance was detected during the action of somatostatin (Dodd and Kelly, 1978). The depolarization of neurones in the supraoptic nucleus of the hypothalamus by ADH is independent of the extracellular concentrations of K^+, Cl^- and Ca^{2+} ions, although depleting the Na^+ ion concentration reduced the response slightly. The depolarization is blocked by ouabain, and is believed to involve a second messenger, possibly cAMP (Abe, Inone, Matsuo and Ogata, 1983).

The action of CCK on pancreatic acinar cells, albeit not on neurones, has been investigated using patch clamp techniques (Maruyama and Petersen, 1982). Application of CCK to an acinar cell induced single channel currents (as recorded with a cell-attached patch) which were inward at resting membrane potentials. These CCK activated channels

were selective for cations, and the single channel current reversed at around -5 mV. CCK, and also ACh, activated these channels via a second messenger, which was shown to be Ca^{2+} ions.

1.2.5 PEPTIDES AS INTERNEURONAL MESSENGERS IN VERTEBRATES.

Most of the above mentioned peptides are strong candidates for transmitters in the mammalian CNS. Snyder (1980) regarded transmitter candidates as those peptides which are located in specific neuronal systems, released on depolarization, and which change neuronal activity. As new neuropeptides are being discovered, many of them fulfil most of the criteria for classification as transmitter substances. Thus synaptic communication in vertebrates is likely to involve many more transmitter compounds than was originally thought.

There is much evidence presented by Otsuka, Konishi, Yanagisawa, Tsunoo and Akagi (1982) and Otsuka and Konishi (1983) which supports a sensory transmitter role for substance P. There is a high concentration of substance P in the dorsal horn which appears to be confined to small diameter C-fibres. Substance P containing nerve terminals have been detected immunocytochemically in the substantia gelatinosa. Stimulating the dorsal roots causes a Ca dependent release of substance P, while sectioning the dorsal roots leads to a reduction of the dorsal horn

content of substance P. The evidence presented above by these authors, together with the depolarizing action of substance P on spinal neurones (see section 1.2.4) is consistent with a neurotransmitter role for substance P in the spinal cord.

Many other neuropeptides have been proposed as transmitter compounds. A neurotransmitter role was suggested for enkephalins by Hughes, Kosterlitz and Smith in 1977. The actions of somatostatin, CCK and VIP, all of which induce a pronounced excitation of CA1 and CA2 neurones in the hippocampus (Dodd and Kelly, 1978; Snyder, 1980), are consistent with a transmitter role for these three peptides. In fact CCK, which is the most abundant brain peptide in mammals, has been proposed to be an important excitatory transmitter in mammalian cerebral cortex and hippocampus (Iversen, Lee, Gilbert, Hunt and Emson, 1980).

1.2.6 DOES PEPTIDERGIC TRANSMISSION OCCUR IN BULLFROG SYMPATHETIC GANGLIA?

There are technical disadvantages associated with the study of synaptic transmission in the mammalian CNS because the large number of small neurones render intracellular recording of synaptic events difficult. The bullfrog sympathetic ganglion, however, is an ideal preparation for the study of synaptic mechanisms because

the recording of postsynaptic potentials following preganglionic nerve stimulation is possible. Following preganglionic stimulation, a four component response is observed - a fast excitatory postsynaptic potential (EPSP), a slow inhibitory postsynaptic potential (IPSP), a slow EPSP and a late slow EPSP. The first three components are blocked by a mixture of nicotinic and muscarinic antagonists (Jan, Jan and Kuffler, 1979), leaving the late slow EPSP unaffected. This late slow EPSP is mimicked by the application of LHRH (Adams and Brown, 1980; Jan, Jan and Kuffler, 1979; Jan and Jan, 1982). There is substantial evidence implying that an LHRH-like peptide is the transmitter mediating the late slow EPSP in bullfrog sympathetic ganglia (Jan and Jan, 1983). Using a radioimmunoassay for LHRH, an LHRH-like peptide has been localised within synaptic boutons encircling bullfrog sympathetic ganglion somata (Jan, Jan and Brownfield, 1980). Stimulation of preganglionic nerves results in a Ca dependent release of this peptide. Furthermore, both the nerve evoked and the LHRH evoked late slow EPSP have the same physiological and pharmacological properties (Jan and Jan, 1982). The results mentioned above are consistent with an LHRH-like peptide being the transmitter mediating the late slow EPSP in bullfrog sympathetic ganglia.

Synaptically released LHRH-like peptide is capable of acting at a distance in the sympathetic ganglion of the bullfrog (Jan and Jan, 1983). If other transmitter substances are capable of acting at a distance, then a way of localizing their action would be to have a topographical distribution of receptor sites on neurones in the vicinity of the presynaptic release site. Therefore, a transmitter would only influence those areas where receptors for that particular transmitter were present (Jan and Jan, 1983). Thus integrative neurocommunication based on this hypothesis would necessitate the existence of many transmitter compounds, and a multitude of receptors, in order to avoid non-specific effects of a released transmitter.

1.3 NEUROPEPTIDES IN INVERTEBRATES.

1.3.1 ISOLATION AND LOCALIZATION OF INVERTEBRATE PEPTIDES.

The identification and sequencing of invertebrate peptides is made difficult by the small amount of peptide present in invertebrate nervous systems, and also the possibility that a peptide may only be present in a few identified neurones. Paradoxically, one of the first peptides to be sequenced was eledoisin, a substance P-like peptide, isolated from the salivary glands of the octopod Eledone (Erspamer and Anastasi, 1962). The presence of biologically active neuropeptides has often been indicated by an action of peptide containing ganglion extracts on neurones or muscles (Brown and Starratt, 1975; Ifshin, Gainer and Barker, 1975; Levitan and Treistman, 1977; Lloyd, 1978). With the advancement of isolation and purification techniques, such as high performance liquid chromatography, some 17 native invertebrate peptides have now been isolated and characterized, mainly from arthropods and molluscs. These peptides, together with the animals from which they were isolated, are listed in Table 1:1. The arthropod neuropeptide proctolin (Brown and Starratt, 1975), the egg laying hormones of Aplysia (Chiu, Hunkapillar, Heller, Stuart, Hood and Strumwasser, 1979; Heller, Kaczmarck, Hunkapillar, Hood and Strumwasser, 1980; Rothman, Mayeri, Brown, Yuan and

Table 1:1. A list of neuropeptides discovered from invertebrates. This list has been obtained from two reviews of Greenberg and Price (1983) and O'Shea and Schaffer (1985).

PEPTIDE	ANIMAL
Proctolin	*Periplaneta americana*
Red pigment concentrating hormone	*Pandalus borealus*
Distal retinal pigment hormone	*Pandalus borealus*
Paragonial peptide	*Drosophila funebris*
FMRFamide	*Macrocallista nimbosa*
pQDPFLRFamide	*Helix aspersa*
Small cardioexcitatory peptide	*Aplysia brasiliana*
Egg-laying hormone	*Aplysia californica*
Egg-releasing hormone	*Aplysia californica*
α-bag cell peptide	*Aplysia californica*
β-bag cell peptide	*Aplysia californica*
Peptide A	*Aplysia californica*
Peptide B	*Aplysia californica*
R3 - R14 peptide	*Aplysia californica*
Eledoisin	*Eledone moschata*
Hydra head activator	*Anthopleura elegantissima*

Shively, 1983) and the molluscan neuropeptide FMRFamide (Price and Greenberg, 1977) are amongst the most studied invertebrate neuropeptides.

The discovery of neuropeptides in mammalian CNS prompted a search for these neuropeptides in invertebrate nervous systems. Immunoreactive staining of invertebrate neurones has been observed using antisera raised against many peptides including substance P, somatostatin, TRH and opioid peptides (Haynes, 1980).

1.3.1.1 ISOLATION AND CHARACTERIZATION OF FMRFamide.

Extracts of molluscan ganglia are well known to contain peptide factors which are cardioactive (Agarwal and Greenberg, 1969), and which may alter neuronal activity (Ifshin, Gainer and Barker, 1975). This activity is most pronounced in a chromatographic fraction termed 'peak C' (Frontali, Williams and Welsh, 1967), which is found in almost all molluscan species which have been studied (Greenberg and Price, 1979). Using a parallel bioassay with the Busycon radula protractor muscle and the heart of the clam Mercenaria mercenaria, peak C was isolated from ganglia of the clam Macrocallista nimbosa, and purified using a combination of solvent extractions, gel-filtration and ion-exchange chromatography (Price and Greenberg, 1977). The structure of peak C was determined as an amidated tetrapeptide, Phe-Met-Arg-Phe-NH_2

(FMRFamide), by amino acid analysis and the comparison of the effect of the purified peptide with the sequenced peptide on the bioassay systems (Price and Greenberg, 1977). The abbreviation, FMRFamide, is derived from the single letter nomenclature for amino acids (IUPAC-IUB, 1972; see also abbreviations). Single letter abbreviations will be used frequently throughout this thesis.

1.3.1.2 DISTRIBUTION OF FMRFamide WITHIN THE ANIMAL KINGDOM.

As has already been mentioned, chromatographic peak C is found in most molluscs. However, although the extracted fraction may have FMRFamide-like biological activity, the molecule itself may not be FMRFamide but a closely related structure. Using antisera raised against FMRFamide, immunoreactive FMRFamide has been found in many vertebrate and invertebrate phyla (Boer, Schot, Veenstra and Reichelt, 1980; Dockray, Vaillant and Williams, 1981; Schot and Boer, 1982). The identity of a peptide cannot be based solely on immunocytochemical evidence as is demonstrated by the specificity curves for the antisera raised against FMRFamide by Weber, Evans, Samuelson and Barchas (1981). These authors demonstrated a novel peptide system in the rat CNS which was detected using antibodies to FMRFamide. Although the antibodies were raised against FMRFamide, they showed that the sequence

-RFamide was the minimum recognition requirement for the radioimmunoassay.

A number of peptides throughout the animal kingdom may be related to FMRFamide. Chicken brain contains a large amount of FMRFamide immunoreactive peptide. Purification and characterization of this peptide was performed by Dockray, Reeve Jr., Shively, Gayton and Barnard (1983). The structure was deduced as Leu-Pro-Leu-Arg-Phe-NH_2 (LPLRFamide). Recently Dockray (1985) has characterized FMRFamide-like immunoreactivity in the rat spinal cord using three antisera in association with HPLC purification techniques. Two of the antisera were raised against FMRFamide, and the third was raised against Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH_2 (YGGFMRFamide). None of the major extracts correspond to FMRFamide, YGGFMRFamide or LPLRFamide. A possible sequence is -M(or L)RFamide with an N-terminal extension. Application of FMRFamide to mammalian neurones has been shown to produce excitatory and inhibitory responses (Gayton, 1982; McCarthy and Cottrell, 1984), and LPLRFamide has similar actions as FMRFamide in the rat (Dockray, Reeve Jr., Shively, Gayton and Barnard, 1983). These results demonstrate that certain mammalian and other vertebrate neurones contain FMRFamide-like peptides, and that some vertebrate neurones have the capacity to recognize and respond to FMRFamide and related peptides.

1.3.1.3 FMRFamide PEPTIDES IN *HELIX*.

Cottrell, Price and Greenberg (1981) demonstrated the presence of FMRFamide-like biological activity in extracts of *Helix* ganglia. When the *Helix* peptide was co-chromatographed with FMRFamide, the peaks containing biological activity eluted at different times. Furthermore, the purified peptide extract has 600 times more cardioexcitatory activity than FMRFamide, as calculated using *Helix* heart and *Busycon* radula protractor muscle as a parallel bioassay (Cottrell, Price and Greenberg, 1981). This suggested that the FMRFamide-like peptide of *Helix* was not FMRFamide.

In a later study involving the pooled extracts from 800 ganglia, several peaks of immunoreactivity were detected (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman and Riehm, 1985). In contrast to previous reports, FMRFamide itself was found. The most abundant peptide, and the first analogue of FMRFamide to be extracted from molluscs, has the structure pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (pQDPFLRFamide). Other FMRFamide related peptides are present in *Helix* and also in the ganglia of other snails. Purified FMRFamide peptides from *Lymnaea* are FLRFamide, GDPFLRFamide and SDPFLRFamide (D.A. Price, personal communication). It is believed that one or both of the latter two are present in *Helix*. Interestingly,

the FMRFamide peptide of the related mollusc, Aplysia, is FMRFamide (Lehman, Price and Greenberg, 1984).

FMRFamide peptides have also been detected immunocytochemically in Helix. An identified neurone, the C3 neurone in the cerebral ganglion shows positive immunoreactive staining when exposed to antisera raised against FMRFamide (Cottrell, Schot and Dockray, 1983). The processes of this cell were visualized following intracellular injection of Lucifer Yellow. Axonal branches of the C3 neurone were followed into the tentacle retractor muscle, where many anti-FMRFamide reactive neuronal processes are embedded within the muscle fibres. It is uncertain whether these peptidergic fibres in the muscle originate from the C3 neurone. Electrical stimulation of the C3 neurone causes the tentacle retractor muscle to contract. This system appears to be very similar to the proctolinergic system described in Periplaneta by O'Shea and Bishop (1982) (see section 1.3.5).

1.3.2 RELEASE OF NEUROPEPTIDES IN INVERTEBRATES.

Investigations of the release of neuropeptides from invertebrate neurones is made difficult because of the very small amount that is released. The release of proctolin from an identified neurone in Periplaneta has been demonstrated by Adams and O'Shea, (1983). The

release could be induced by stimulating the Ds neurone, or by depolarizing nerve terminals of the Ds neurone with a high K solution. The Ds neurone innervates the coxal depressor muscle of Periplaneta. The release of proctolin from a nerve muscle preparation was detected by comparing HPLC fractions of the released peptide with that of synthetic proctolin. The released peptide co-eluted exactly with tritiated synthetic proctolin. This release was dependent on the presence of Ca, and was blocked during exposure of the preparation to 5 mM Co.

The bag cell peptides of Aplysia are released following a brief electrical stimulus given to the bag cells (Scheller, Rothman and Mayeri, 1983). The release of these peptides affect many neurones throughout the abdominal ganglion of Aplysia (see below), indicating that some of the released peptides act more as local hormones than synaptic transmitters.

1.3.2.1 SUBCELLULAR LOCALIZATION AND RELEASE OF FMRFamide.

The localization of FMRFamide at the subcellular level has been studied in the clam Macrocallista by a differential and density gradient fractionation method (Nagle, 1981). Macrocallista ganglia were homogenized and taken through a series of centrifugation steps. The highest relative specific activity of FMRFamide, as tested with a bioassay method, occurred in the microsome

containing pellet. Centrifugation on a discontinuous sucrose density gradient resulted in the highest FMRFamide activity occurring in a fraction containing neurosecretory granules with a diameter of about 100 nm. Neurosecretory granules were also observed in electron micrographs of intact pedal ganglia (Nagle, 1981).

Using a radioimmunoassay to detect FMRFamide, a Ca dependent release of FMRFamide has been shown to occur from Macrocallista ganglia depolarized by exposure to high K solution (Nagle, 1982). The high K induced release of FMRFamide was inhibited by removing Ca from the bathing solution. The association of FMRFamide with neurosecretory granules, and the Ca dependent release following depolarization is strong evidence supporting FMRFamide as a neurosecretory product in Macrocallista.

In the pond snail Lymnaea, there is ultrastructural evidence for the release of FMRFamide-like peptides. In the soma of E-cells, numerous FMRFamide reactive granules exist (Boer, Schot, Reichelt, Brand and ter Maat, 1984). E-cell axons which make contact with the smooth muscle fibres in the penis retractor muscle contain similar granules. Other axons which contain FMRFamide reactive granules tunnel into the cytoplasm of the "light yellow associated cell" (LYAC). The sites of close contact between the E-cell terminals and the muscle, and between the FMRFamide reactive fibres and the LYAC are possibly

synaptic junctions (Boer et al., 1984). Interestingly, LYAC is depolarized by FMRFamide. The cytoplasm of the C3 neurone in Helix also contains many peptidergic granules (Cottrell, Schot and Dockray, 1983).

1.3.3 EFFECTS OF NEUROPEPTIDES ON INVERTEBRATE NEURONES.

The detailed investigation of the electrical actions of neuropeptides is restricted to preparations where stable intracellular recordings are possible. In vertebrate nervous systems these are few, although with the advancement of tissue culture techniques and the application of the whole cell configuration of the patch clamp, the data on neuropeptide actions on vertebrate central neurones is likely to expand rapidly. Some invertebrate nervous systems, however, and in particular molluscan nervous systems offer preparations where many of the neuronal somata are large and easily accessible to detailed electrophysiological investigations. Thus most of the results on the neuronal actions of neuropeptides in invertebrates have been obtained from molluscs.

Many mammalian neuropeptides have been applied to molluscan neurones. Application of ADH and oxytocin onto cell 11 of the snail Otala induces a bursting pacemaker potential in this neurone (Barker and Gainer, 1974). The effect is dose dependent and may last for several (30 to 60) minutes. In a voltage clamp investigation of this effect, application of ADH changed the current voltage (I-V) relationship of this neurone (Barker and Smith, 1976). In the absence of peptide, the slope conductance

of this neurone was positive, but during the presence of ADH, a region of negative slope conductance appeared, giving the I-V curve an 'N' shape, which is characteristic of bursting neurones. This effect appeared to be the induction of a Na current, although an effect on a K current was also likely. The related peptide oxytocin has been shown to be a powerful excitant of salivary gland cells of Philine (Barber, 1983).

Other vertebrate peptides which have been shown to affect invertebrate neurones are Met-enkephalin and CCK. In Helix pomatia, the RPa2 neurone is excited by local application of Met-enkephalin from a blunt microelectrode. A marked depolarization occurred which was followed by an increase in action potential frequency (Stefano, S.-Rozsa and Hiripi, 1980). This effect was also induced by morphine, and was antagonized by naloxone. Most of the neurones tested in Helix aspersa were hyperpolarized by 160 nM CCK. This hyperpolarization was sometimes accompanied by a decrease in conductance (Bokisch, Osborne and Walker, 1983).

One of the first reports of an endogenous invertebrate peptide affecting neuronal activity was that by Ifshin, Gainer and Barker in 1975. Peptide extracts from homogenized ganglia induced bursting behaviour in cell 11 of Otala in a similar fashion to ADH (see above). However, the peptide extract was shown to differ

chemically from ADH. The augmentation of bursting behaviour in Helix and Aplysia neurones by ADH and oxytocin was also mimicked by a crude peptide containing extract of molluscan ganglia (Levitan and Treistman, 1977; Levitan, Harmar and Adams, 1979). The application of isobutyl methyl xanthene (IBMX) also augmented the bursting behaviour. It was shown that the peptide extract increased the cellular levels of both cAMP and cGMP in these neurones, indicating that the effect of the peptide extract was mediated by cyclic nucleotides.

The egg laying hormone, released following stimulation of the bag cells in the abdominal ganglion of Aplysia, affects many neurones in this ganglion. The effects include an increase in the bursting pacemaker potential of the R15 neurone, an abolition of the bursting activity of L3 and L6, a slow inhibition of R2, R3-14 and L14A, L14B and L14C, a transient excitation of L1 and R1 and a prolonged excitation of LB and LC (Mayeri, Brownwell, Branton and Simon, 1979; Mayeri, Brownwell and Branton, 1979). The other peptides which are released from the bag cells, and which have similar multiple actions are α- and β-bag cell peptides (Scheller, Rothman and Mayeri, 1983). These actions of the peptides, released following bag cell stimulation, may last for several hours.

1.3.3.1 EFFECTS OF FMRFamide PEPTIDES ON INVERTEBRATE NEURONES.

FMRFamide, the first molluscan cardioactive peptide to be sequenced, was shown to be neuroactive by Cottrell, who described a hyperpolarizing response (Cottrell, 1978) and a voltage dependent depolarising response (Cottrell, 1979) in neurones of Helix pomatia. In a later study on Helix aspersa neurones, the hyperpolarization was shown to result mainly from an increase in gK, while the voltage dependent response was the result of a decrease in an outward K current (Cottrell, 1982a). The C1 neurone in each cerebral ganglion responds with a combination of these two conductance changes. YGGFMRFamide also induced these two responses, although the heptapeptide was more potent than FMRFamide at producing the voltage dependent response in the C1 neurone. The increase in gK was reduced by 30 mM TEA, while the voltage dependent decrease in gK was blocked by Co^{2+} and Ba^{2+} ions, but augmented following intracellular injection of Ca (Cottrell, 1982a). These results were consistent with the voltage dependent response resulting from a suppression of a Ca activated K current, which is known to occur in molluscan soma (Adams, Smith and Thompson, 1980). Application of single channel recording methods to study this response revealed that the activity of K channels recorded by the cell attached patch method was decreased following

administration of FnLRFamide (a FMRFamide analogue) to the cell membrane outwith the patch (Cottrell, Davies and Green, 1984). Recently, FMRFamide has been shown to decrease both the Ca current and a cAMP dependent K current in neurones of the suboesophageal ganglia of Helix (Colombaioni, Paupardin-Tritsch, Vidal and Gerschenfeld, 1985). This is in agreement with the single channel data presented by Cottrell, Davies and Green (1984), which suggests the involvement of a second messenger in the production of the voltage dependent response. In addition to the increase in gK, and the voltage dependent decrease in gK, a depolarization resulting from an increase mainly in gNa has been shown to occur in these neurones. This response is rapidly desensitized by repeated application of FMRFamide (Cottrell, Davies and Green, 1984; Boyd and Walker, 1985).

1.3.3.2 EFFECTS OF FMRFamide PEPTIDES ON VERTEBRATE NEURONES.

FMRFamide has been shown to affect mammalian neurones both in vivo and in vitro. Application of FMRFamide predominantly excited rat brain stem neurones, while the related opioid peptide YGGFMRF had a depressant action (Gayton, 1982). The depressant effect of YGGFMRF was antagonized by naloxone, however, the action of FMRFamide was unaffected by naloxone. The related peptide LPLRFamide, isolated from chicken brain, has similar

actions to FMRFamide on brain stem neurones (Dockray, Reeve, Shively, Gayton and Barnard, 1983). Recently it has been reported that both FMRFamide and FMRFamide immunoreactive material isolated from bovine brain can reduce the analgesia produced in rats by YGGFMRF and morphine (Tang, Yang and Costa, 1984). In an intracellular study of cultured mouse spinal neurones, two types of responses to FMRFamide were observed, a depolarization associated with a decrease in conductance and a response associated with an increase in conductance (McCarthy and Cottrell, 1984). The first response had properties consistent with a decrease in gK mechanism, while the later response had a variable reversal potential, ranging from -70 to -20 mV, and involved both Na^+ and Cl^- ions.

1.3.4 PEPTIDES AS INTERNEURONAL MESSENGERS IN INVERTEBRATES.

The organization of some invertebrate nervous systems, particularly those of molluscs and arthropods, offer preparations where many individual neurones and their follower cells can be identified. This is an obvious advantage over most vertebrate CNS preparations for the study of neurotransmission. However, biochemical analysis of invertebrate peptide mechanisms, such as release from neurones, is made very difficult due to the small amount of peptide which is released from possibly

very few nerve terminals. There are, however, invertebrate peptide systems which offer a unique opportunity to investigate the physiology of neuropeptides in invertebrates.

Examples of identified peptidergic neurones in invertebrates occur in Aplysia, Helix and Periplaneta. Numerous peptides are released from the bag cells of Aplysia which appear to act as local hormones involved in the control of egg-laying. The C3 neurone of Helix contains immunoreactive FMRFamide. (Cottrell, Schot and Dockray, 1983). This neurone innervates the tentacle retractor muscle of Helix. Electrical stimulation of this neurone causes a contraction of the tentacle retractor muscle, however, it is unclear whether this contraction arises due to the release of a FMRFamide-like peptide. Another example of an identified peptidergic system in invertebrates is the innervation of the coxal retractor muscle by the Ds neurone in Periplaneta. This example is discussed below.

1.3.5 AN EXAMPLE OF A PEPTIDERGIC SYSTEM IN INVERTEBRATES.

Immunoreactive proctolin has been found in the CNS of Periplaneta, in particular there is a symmetrical pair of large, proctolin-immunoreactive cell bodies in the metathoracic ganglion (O'Shea and Bishop, 1982). These neurones could be identified from preparation to

preparation, and their anatomical features, observed following intracellular injection of the fluorescent dye Lucifer Yellow, are consistent with those of insect motoneurones. Both crude and purified extracts from the isolated proctolin-immunoreactive cell bodies had biological activity similar to that of authentic proctolin. Furthermore, the extract co-migrates with proctolin during reverse phase liquid chromatography, suggesting that the proctolin-like substance in these neurones is indeed proctolin (O'Shea and Bishop, 1982). These neurones (termed the Ds neurones) innervate the coxal depressor muscle, and proctolin-immunoreactive terminals exist within this muscle. Extracts of the coxal depressor muscle have proctolin-like biological activity (Adams and O'Shea, 1983). In a nerve-muscle preparation, electrical stimulation of Ds axons, or exposure of the preparation to high K caused the release of proctolin. This release was Ca dependent (Adams and O'Shea, 1983). Stimulation of the Ds motoneurone produces contractile responses in the coxal depressor muscle. A single action potential yields an excitatory junction potential, followed by a transient contraction, typical of the action of glutamate which is believed to be an insect neuromuscular transmitter. A burst of action potentials, however, produces a biphasic contraction, the transient contractions still exist, but there is a prolonged contraction which is very similar to the effect of proctolin on the muscle. These results demonstrate that

proctolin is released as a cotransmitter with another substance, which is probably glutamate, from the Ds neurones (Adams and O'Shea, 1983).

In this system the invertebrate neuropeptide proctolin has been shown to exist in two identified motoneurones which innervate the coxal depressor muscle of Periplaneta. Proctolin is released from this muscle in a Ca dependent manner following depolarization, and has a contractile effect on the muscle. This is a good example of a neuropeptide acting as a transmitter substance in invertebrates. Studies on other neuropeptide systems need to be undertaken to determine whether non-peptide transmitters are generally co-released with peptides.

1.4 PHARMACOLOGY OF NEUROPEPTIDES.

Most of the work on neuropeptide receptors has been done on mammalian central and peripheral nervous systems. The receptors have been characterized mainly by radioligand binding experiments in CNS, and by parallel bioassay methods using peripheral tissue such as guinea pig ileum and rat vas deferens. A relatively small amount of work has been done on invertebrate peptide receptors. Because of the analogy observed between FMRFamide and opioid systems, the pharmacology of endogenous opioid peptides will be briefly discussed as an example of a neuropeptide system comprising of multiple receptors and agonists.

1.4.1 PHARMACOLOGY OF OPIOID PEPTIDES.

Three opioid precursors have been characterized which between them give rise to some 18 opioid peptides following differential proteolytic processing of the precursor molecules (Weber, Evans and Barchas, 1983). The resulting opioid peptides show different pharmacological profiles with different affinities towards the types of opioid receptors.

The discovery that opioid receptors consisted of different types was facilitated by the availability of antagonists, some of them such as β-funaltrexamine being highly selective (Goldstein and James, 1984). Since the discovery of the two endogenous opioid ligands, Met- and Leu-enkephalin, a large number of endogenous ligands, have been discovered. The affinity of these various endogenous opioid ligands towards the four main receptor types are very different. All of them interact to varying degrees with the different receptor types (Kosterlitz, 1985).

The pharmacological activities of opioid peptides have been investigated using five bioassay systems:- the mouse vas deferens (μ-, δ- and k-receptors), guinea-pig ileum (μ and k), rabbit vas deferens (k), hamster vas-deferens (δ) and rat vas deferens (μ and ϵ). The types of receptors on these tissues have been demonstrated using selective peptide and non-peptide opioid analogues (Kosterlitz, 1985). Using these bioassay preparations, it appears that the minimum requirement for opioid activity is the N-terminal sequence Tyr-Gly-Gly-Phe (i.e. amino acids 1-4). This sequence has been termed the message component of the peptide, while amino acids 5 onwards is termed the address (Chavkin and Goldstein, 1981).

A group of endogenous opioids arise from the prodynorphin precursor molecule. The guinea-pig ileum was used as a bioassay system to test these dynorphin gene products for their affinity towards μ- and k-receptors by James, Fischli and Goldstein (1984). Their results are tabulated below for clarity:

		RECEPTOR	
		k	μ
dynorphin (1-8)	YGGFLRRI	0.0034	PA
β-neoendorphin	YGGFLRKYP	0.0048	PA
α-neoendorphin	YGGFLRKYPK	0.073	
dynorphin B	YGGFLRRQFKVVT	0.093	
dynorphin A	YGGFLRRIRPKLKWDNQ	1.000	
N-terminal	1-----7---11-----	C-terminal	

The potency of the peptides towards k-receptors is expressed relative to a value of 1.0 for dynorphin A. Both dynorphin (1-8) and β-neoendorphin are partial agonists (PA) at μ-receptors. However, α-neoendorphin, dynorphin B and dynorphin A are highly selective agonists. Thus extending the peptides at the C-terminal alters the relative potency towards μ- and k-receptors. It appears, however, that basic residues in position 7 and also 10 or 11 are essential for k-receptor selectivity (James, Fischli and Goldstein, 1984). Interestingly, Leu-enkephalin (i.e. YGGFL) has very little affinity for

κ-receptors, but interacts appreciably with μ-receptors.

A similar situation occurs with the affinity of different fractions of β-endorphin (from the pro-opiomelanocortin precursor) for the ϵ-receptor in the rat vas deferens. This receptor type has a high selectivity for β-endorphin and shows a lack of affinity for the enkephalins (Schulz, Wuster and Herz, 1981). The affinity of β-endorphin and its fragments towards δ-, μ- and ϵ-receptor types was tested by selectively removing single amino acids from the C-terminal. Starting from β-endorphin (1-31) the affinity towards all three receptors was similar. However, on reaching the fragment 1-21, the affinity towards the ϵ-receptor decreased markedly, while the affinity towards the other two receptors remained virtually unaltered (Schulz, Wuster and Herz, 1981).

1.4.2 PHARMACOLOGY OF FMRFamide PEPTIDES.

FMRFamide has been tested on a wide variety of molluscan cardiac and non-cardiac muscles. Both 5-HT and FMRFamide increase the rate and force of contraction of Mercenaria heart. However, the cardioexcitatory effect of 5-HT is blocked by methysergide whereas that of FMRFamide is not. In addition, the contraction of the Busycon radula protractor muscle induced by ACh is blocked by

benzoquinonium, a compound which has no effect on the FMRFamide evoked contraction of this muscle (Price and Greenberg, 1980). When various FMRFamide analogues were tested on the radula protractor muscle, many of them had very little or no biological activity. From these results it appears that there are specific receptors for FMRFamide on molluscan muscles.

A survey has been conducted on the actions of FMRFamide analogues on the Busycon radula protractor muscle. The biological activity of a number of analogues, related to a value of 1 for FMRFamide, is listed below:

	relative activity
FMRFamide	1
MRFamide	<0.001
RFamide	<0.0001
FMRF	0
MRF	0
RF	0
t-Boc-MRFamide	0.02

These results from Price and Greenberg (1980), show that the C-terminal amide is essential, and that activity is rapidly lost through removal of N-terminal amino acids. This study has been extended to include the effects of

such analogues on four bioassay tissues. These are Macrocallista heart, Lampsilis heart, Busycon radula protractor muscle and Geukenia anterior byssus retractor muscle (ABRM) (Painter, Morley and Price, 1982). From this survey, using these four bioassay tissues, it was evident that the biological activity of an analogue was not changed in a parallel fashion on all of the tissues. For example, the relative activity of YGGFMRFamide on Geukenia ABRM was 1.4 while the activity on the Busycon radula protractor was only 0.3. Similarly, the relative activity of YMRFamide on Lampsilis heart was 0.4 and on the ABRM it was only 0.02 (Painter, Morley and Price, 1982). The requirements for biological activity are, however, similar on all four of these muscles, but the fact that the relative activity of some analogues differ depending on the muscle used indicates that the FMRFamide receptors may be different.

The opioid related peptide YGGFMRFamide is a potent analogue of FMRFamide on the above preparations, having qualitatively similar actions as FMRFamide itself (Greenberg, Painter and Price, 1981). On some molluscan muscles, however, YGGFMRFamide and FMRFamide have differential actions, notable examples being the tentacle retractor muscle of Helix, and the ABRM of Mytilus. In Mytilus ABRM, both FMRFamide and YGGFMRFamide produced contractions. At concentrations between $1x10^{-8}$ and $1x10^{-7}$M, FMRFamide relaxed catch contractures.

YGGFMRFamide did not relax catch contractures at any concentration tested (Muneoka and Matsuura, 1985). FMRFamide induces a rhythmic contraction of the Helix tentacle retractor muscle. The threshold concentration for this effect was about $5x10^{-10}$ M, with the maximum effect occurring at around $1x10^{-6}$ M. A further increase in the concentration of FMRFamide resulted in a mixture of excitatory and inhibitory actions (Cottrell, Greenberg and Price, 1983). Low concentrations of YGGFMRFamide (below $1x10^{-8}$ M) also induced rhythmic contractions in this muscle. However, at concentrations higher than $2x10^{-8}$ M, YGGFMRFamide induced pronounced inhibitory effects, and could reduce or abolish the contractions produced by FMRFamide and ACh.

Differential effects of FMRFamide and YGGFMRFamide have also been reported on the C1 neurone of each cerebral ganglion of Helix (Cottrell, 1982a). In the investigation presented here, I report further differential effects of FMRFamide analogues on Helix neurones and provide evidence supporting the presence of multiple FMRFamide receptors on Helix neurones.

1.5 CONCLUSIONS AND OBJECTIVES.

Neuropeptides have been isolated and characterized from the nervous systems of a number of animals of both vertebrate and invertebrate phyla. Many of these neuropeptides have been shown to have potent and diverse actions on individual neurones. Furthermore, there is evidence supporting the release of these peptides in a Ca dependent manner from nerve terminals, thus suggesting an interneuronal messenger role.

This thesis describes the electrophysiological actions of the molluscan neuropeptide FMRFamide and related peptides on neurones of the garden snail Helix aspersa. The discovery of FMRFamide and a related peptide (pQDPFLRFamide) in Helix suggests that these peptides may have an important neurophysiological role in this animal, and that this preparation could provide a valuable insight into endogenous neuropeptide mechanisms. Some of the results presented in this theses have been published (Cottrell and Davies, 1985; Cottrell, Davies and Green, 1984; Davies, 1984).

CHAPTER 2

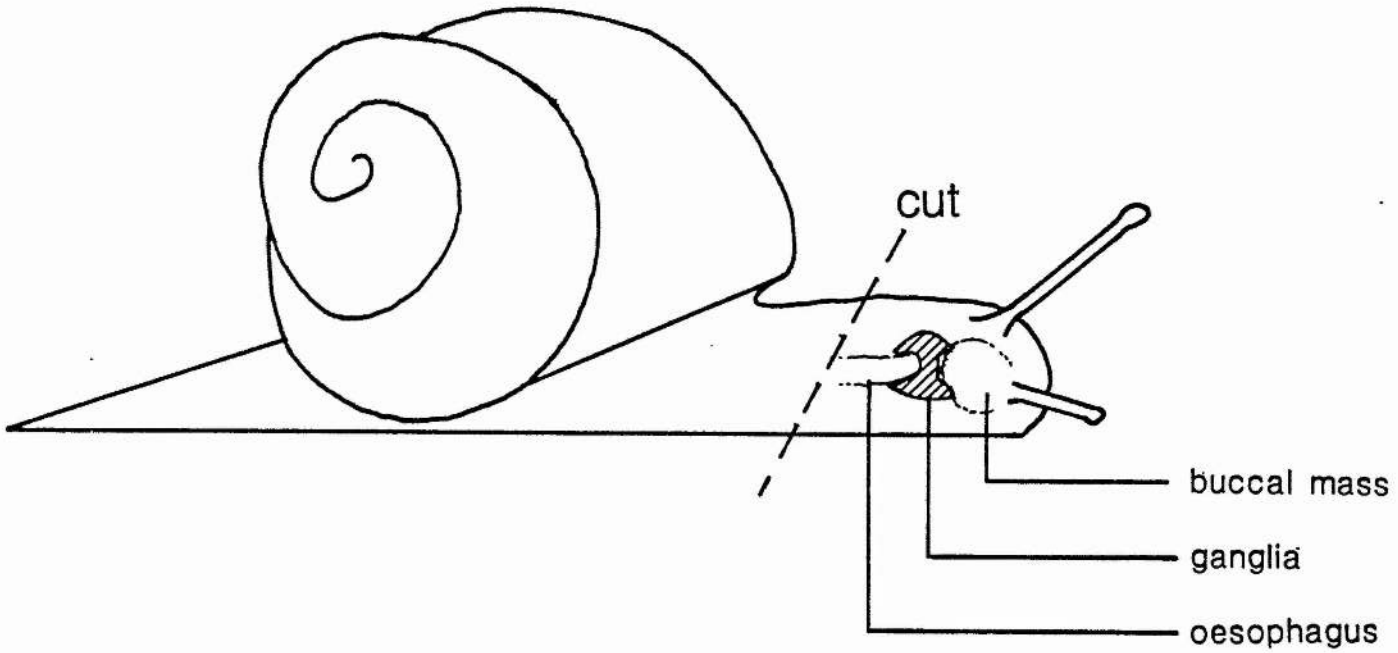
METHODS

2.1 PREPARATION.

Experiments were performed on isolated ganglia from the garden snail Helix aspersa. The animals were collected locally and kept at room temperature until required. Prior to dissection the snails were enticed out of their shells by being placed in water at a temperature of 20 to 30 °C. An active snail was placed on a wax dissecting tray and allowed to crawl over the surface. With a pair of sharp scissors the head was rapidly cut off and pinned to the wax through the mouth (Fig.2:1). This made the ganglia clearly visible, encircling the oesophagus (circumoesophageal ring). The circumoesophageal ring was removed and pinned to the Nylon base of a recording chamber which was filled with physiological solution.

The ganglia are covered with two layers of connective tissue which must be removed before the neurones can be impaled with microelectrodes. This was done by careful microdissection with fine forceps. The outer connective sheath has a spongy texture and was relatively easy to remove. However, the inner sheath is thin and transparent, and had to be carefully ripped apart to expose the neurones. The preparation was viewed through a binocular dissecting microscope (Nikon).

Fig.2:1. Diagram of an active snail showing the position of the circumoesophageal ganglia in relation to the buccal mass and oesophagus. The body of the snail was cut with a pair of scissors at the position indicated by the broken line.


cut
buccal mass
ganglia
oesophagus

2.2 THE RECORDING CHAMBER.

The recording chamber was a rectangular block of Perspex, into which an indentation was cut, forming a small bath about 4mm deep. A thin Nylon sheet was placed in the bath to make a suitable base into which dissecting pins could be inserted. The bath was illuminated by means of a glass rod, one end of which was placed in a rubber bung and connected to a conventional dissecting lamp, the other end was drawn into a thin extension and positioned in the bath below the level of the solution (Fig.2:3). When the tip of the glass rod was placed close to the preparation, adequate illumination was obtained to make the individual soma of the desheathed part of the ganglia clearly visible.

2.3 THE PERFUSION SYSTEM.

The preparation could be exposed to various solutions by means of a multi way tap, to which a maximum of six reservoirs could be connected (Fig.2:2). The reservoirs consisted of large (100ml) syringes connected to smaller (2ml) syringes. Thin plastic tubing, attached to the small syringes, formed the inflows into the six way tap. The outflow of the tap led into the recording chamber. The reservoirs were about 0.4m higher than the recording chamber, thus providing the small driving force necessary

for the flow of solution. The solution from a particular reservoir could be used to perfuse the recording chamber by turning the tap to the desired position. A small clamp connected to the outflow from the tap made it possible to adjust the rate of flow of the perfusate. The rate of flow could be checked by observing the drips occurring as the solution passed from the large syringe into the small syringe.

A suction system disposed of the waste perfusate. The suction tube had to be carefully positioned to maintain the solution in the bath at the desired level, about 1mm above the ganglia. The vacuum was produced by connecting the suction tube to an aspirator via two conical flasks, the first of which collected the waste.

2.4 SOLUTIONS.

Solutions which were commonly used to perfuse the bath are listed in Table 1. Initially experiments were performed using solutions buffered with 5mM Tris and adjusted to pH 7.8 with HCl. However, it has been reported that Tris can block the cation channel opened by ACh in Aplysia neurones (Ascher, Marty and Nield, 1978). For this reason the buffer was changed to 20 mM HEPES and the pH adjusted to 7.5 with NaOH (Meech and Thomas, 1977). No difference was observed between results obtained in Tris buffered and HEPES buffered solutions.

Fig.2:2. Diagram of the six way tap and reservoir apparatus used in the perfusion system. Five reservoirs were connected to the tap by placing the plastic tubing from each reservoir into the different inflows of the tap. Only one reservoir is shown here for convenience.

The shaded part of the tap consists of plastic through which a small channel was bored (see dashed line within the shaded part in the diagram). One end of the channel continued into the outflow of the tap. Rotation of the shaded part allowed alignment of the other end of the channel with any one of the inflows into the tap, thus allowing a choice of bathing solutions during the course of an experiment.

Flow of solution was maintained by the hydrostatic pressure present due to the height of the reservoirs above the outflow. The rate of flow of solution, which could be observed as the drip rate in the small syringe below a reservoir, could be adjusted by a clamp on the outflow tube from the tap (see text).

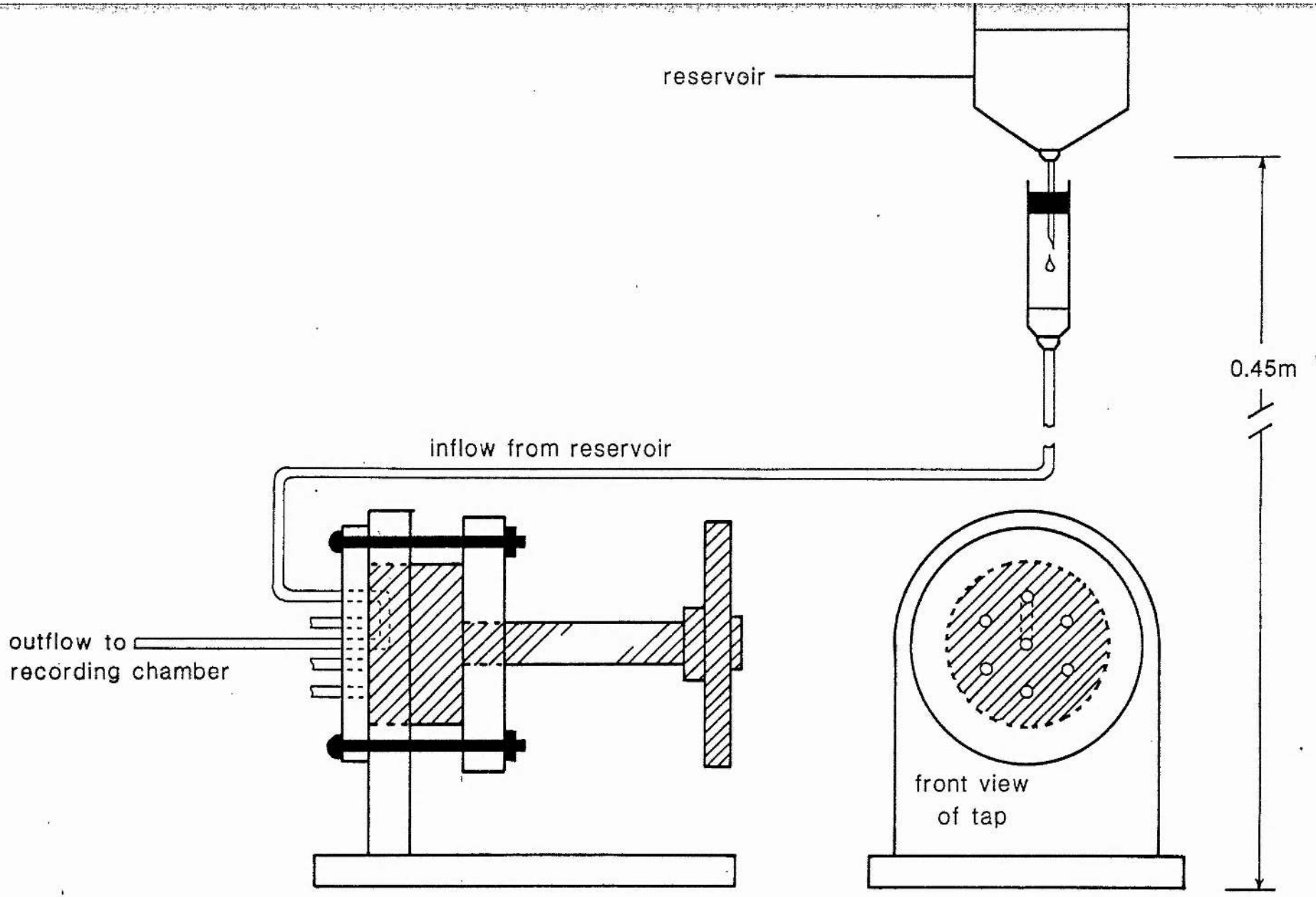

reservoir
0.45m
inflow from reservoir
outflow to
recording chamber
front view
of tap

Table 2:1. The composition of the most commonly used bathing solutions. The concentrations given are in mM. These solutions were adjusted to pH 7.5 by the addition of NaOH. This raised the Na^+ ion concentration by about 10mM. In Tris buffered solutions the concentrations remained the same except that 5mM Tris was used instead of HEPES and the solutions were adjusted to pH 7.8 with HCl (see text).

	NaCl	KCl	$MgCl_2$	$CaCl_2$	TEA	sucrose	HEPES
control	80	5	5	7	0	0	20
low Na	0	5	5	7	0	160	20
TEA test	50	5	5	7	30	0	20
TEA control	50	5	5	7	0	60	20
low K	80	1	5	7	0	0	20
high K	80	15	5	7	0	0	20

In experiments where TEA was used, TEA Br was substituted for NaCl and the control solution had the corresponding amount of sucrose substituted for NaCl to maintain osmolarity. NaCl, KCl, $CaCl_2$, $MgCl_2$, NaOH, tetraethylammonium (TEA) bromide and sucrose were all obtained from BDH Chemicals, HEPES and Tris were obtained from Sigma Ltd.

2.5 RECORDING METHODS.

2.5.1 GENERAL ELECTROPHYSIOLOGICAL METHODS.

Intracellular recordings were made using a single electrode clamp system (Dagan 8100). This made both current and voltage clamp possible with a single microelectrode as described by Wilson and Goldner (1975). Electrical recordings were observed on a dual beam oscilloscope (Tektronix 502A) and permanent records were made on a high quality brush chart recorder (Gould type 220). The current record was filtered before being displayed on the chart recorder to eliminate high frequency oscillations occurring during high gain voltage clamp. This was accomplished by a passive low pass filter using a 160kΩ resistor and a 0.1uF capacitor. This had a cut-off frequency of about 10 Hz. The responses investigated in this study were slow enough not to be distorted by this filter. The bath was grounded with an Ag/AgCl wire connected to the virtual ground of the Dagan

8100 probe. The Ag/AgCl wire was made by dipping a silver wire in molten AgCl. In some experiments where a large change in the ionic composition of the perfusing solution occurred, an agar bridge was connected between the bath and the Ag/AgCl wire to minimize changes in junction potentials. The agar bridge consisted of a tube of glass filled with 3% agar in 3M KCl solution. One end was placed in the recording chamber and the Ag/AgCl wire was pushed into the other end.

2.5.2 ELECTRODES.

Microelectrodes were made using filamented glass capillary tubes of external diameter 1.5mm (Clark Electromedical). They were pulled on a simple vertical electrode puller, using the combination of a spring and gravity to provide the separating force.

Recording electrodes were filled with 2M K acetate solution using a syringe and a fine needle. The filling of the electrodes was assisted by the increased surface tension produced by the filament. The microelectrodes had a resistance of 2 to 3MΩ or less. This low value was required because the recording electrode was also used to pass current (see later). In some experiments K currents were blocked by filling the recording electrode with 2M CsCl solution.

2.5.3 VOLTAGE CLAMP METHOD.

Voltage clamp using a single microelectrode was accomplished by the ability of the amplifier to continuously switch from a current passing mode to a voltage recording mode at a high frequency. Current was injected for 50% of the time while membrane potential was recorded only during the intervals between application of current. The amount of time spent in any one mode had to be substantially less than the time constant of the neurone so that the membrane potential did not have time to wane appreciably, thus the effect is one of simultaneous current injection and voltage recording. The switching frequency was variable, however in most experiments the frequency was set at 3KHz. This method of recording potential and injecting current through the same electrode overcomes the difficulties associated with non-linear current-voltage relationships of microelectrodes.

The negative feedback mechanism of the voltage clamp compared the membrane potential with the desired clamp potential, amplifying the difference using a variable gain (max 5000) amplifier, and adjusting the injected current so as to minimize this difference. The amplifier was also used in a switched current clamp mode.

To enable a neurone to be voltage clamped adequately the electrode had to pass current reliably during the course of the experiment. Electrodes were found to be reliable only if their resistance was less than 2 to 3MΩ. Before impaling a neurone, the resistance of the recording electrode was tested using the impedance test facility of the amplifier. Electrodes with resistances greater than 3MΩ were discarded.

The recording electrode was held to a micromanipulator by a steel spring (see Fig.2:3). The spring was connected to the driven shield of the input probe, thus reducing the stray capacitance of the microelectrode. The gain of the negative feedback amplifier could be set close to the maximum of 5000 using electrodes with resistances of about 1M . However, if too high a gain was used, or the electrode resistance was too high, the clamp would oscillate. The negative capacity compensation was set to a minimum.

2.5.4 LIMITATIONS OF THE VOLTAGE CLAMP.

Electrodes were inserted into the soma of neurones. To check the space clamp of the soma a second recording electrode was placed in the soma. This electrode was connected to a DC preamplifier (Neurolog NL102) and was used to record the membrane potential of the soma during

voltage clamp. Superimposed traces of the voltage from both amplifiers during a command pulse to -20mV from a holding potential of -50mV are shown in Fig.2:4A. This record is from the F1 neurone, one of the largest in the snail. There is good agreement between the potential recorded by the two electrodes, indicating that the space clamp achieved in the soma was good at these potentials. Steady state voltage clamp resulted in good space clamp of the soma up to holding potentials of -10mV. The fidelity of the voltage clamp was not very good. However, in the research conducted here, no fast voltage clamp work was performed.

2.6 APPLICATION OF PEPTIDES.

Peptides were applied either by ionophoresis, pressure ejection, or, in a limited number of experiments, by bath application. The tetrapeptides FMRFamide, FLRFamide and FIRFamide have a positive charge when dissolved in distilled water and could be ionophoresed. YGGFMRFamide could also be ionophoresed but not as well as the tetrapeptides. All other peptides used were applied by pressure ejection.

Fig.2:3. The arrangement of the recording chamber showing the position of the light source, the agar bridge and the recording electrode. In many experiments the agar bridge was omitted and the Ag/AgCl wire was placed directly into the recording solution.

The steel spring surrounding the electrode formed an extension of the driven shield of the probe. The electrode holder was connected to a Narishige micromanipulator. Also shown are the inflow and suction tubes for the bathing solution which are connected to adjustable mounts.

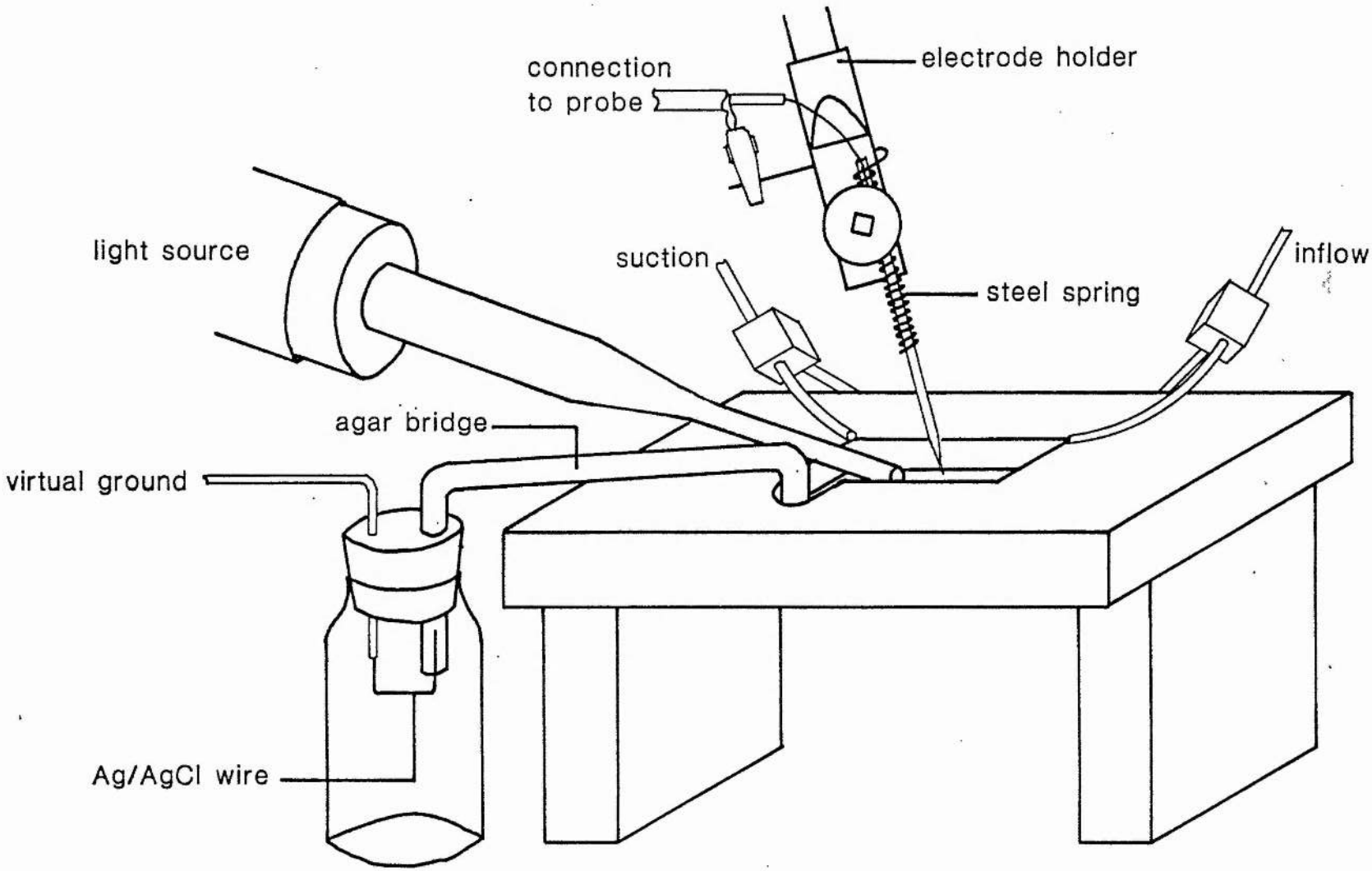
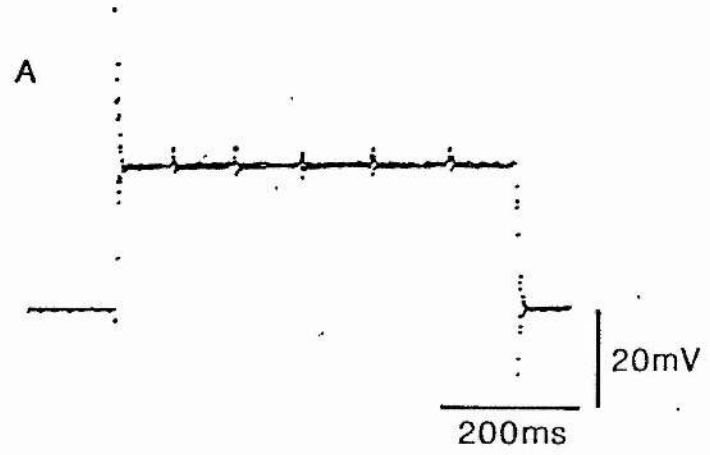
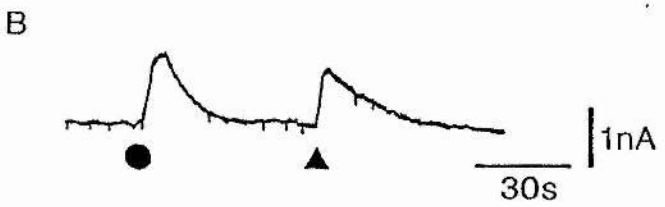

connection
to probe
electrode holder
light source
suction
steel spring
inflow
agar bridge
virtual ground
Ag/AgCl wire

Fig. 2:4. A, testing the space clamp of the Dagan single electrode clamp. A second electrode connected to a DC preamplifier was used to monitor the membrane potential independently of the potential recorded by the voltage clamp electrode in an F1 neurone. The holding potential was -50mV and a 600ms command potential to -20mV was applied. The superimposed voltage record from each electrode showed little deviation from each other during this protocol, suggesting that at these potentials the soma is uniformly voltage clamped. The spikes occurring during the depolarizing command are probably unclamped action potentials originating in the axon. B, comparison of the response elicited by application of FMRFamide by ionophoresis and pressure ejection in a visceral ganglion neurone. ●, ionophoretic application - 200nA for 4s; ▲, pressure ejection - 140kPa for 450ms.


A
20mV
200ms


B
1nA
30s

2.6.1 IONOPHORESIS.

Ionophoresis was performed using a microiontophoresis programmer (WPI Model 160). Ionophoretic pipettes were filled with a 10mM solution of peptide in distilled water. The electrodes tended to block easily. Blocking could be detected by the appearance of a large artefact during ejection and by the decrease in the ejection current. To overcome this, ionophoresis electrodes of low resistances had to be used and were thus pulled on a similar setting as recording electrodes. The resistance of ionophoretic pipettes filled with 10mM peptide solution was about 10MΩ. This corresponded to a resistance of 1MΩ when the electrodes were filled with 2M K acetate.

A reference electrode for the ionophoresis was filled with 2M K acetate solution and placed a small distance away from the ionophoretic electrode. This arrangement isolated the ionophoresis circuitry from the recording circuitry and reduced the ionophoretic artefact. Ejection currents were in the range of 100 to 200nA, and ejection times of about 3 to 5s were used. A retaining current of around 10nA was used in most cases. Leakage of peptide from the pipette could be sufficient to cause a response when the pipette was brought close to the cell. If increasing the retaining current to 20nA did not alleviate this leak, a new ionophoretic pipette was made. A

compromise had to be reached such that the resistance of the ionophoretic pipette was low enough to pass sufficient ejection current, yet high enough so that peptide would not leak out. During the course of the experiments, intervals between peptide ejection were kept as constant as possible to reduce any effect that the retaining current might have on the concentration of peptide at the tip of the ionophoretic pipette.

2.6.2 PRESSURE EJECTION.

Peptides were also locally applied to the soma by pressure ejection from micropipettes. These pipettes had a slightly wider tip than recording or ionophoresis electrodes and were filled with a 1mM solution of peptide in physiological solution. Pressure pulses were timed by a Picospritzer (General Valve Corp. model 2), the amount ejected increasing with pressure and ejection time (Sakai, Swartz and Woody, 1979). Pressure was supplied by a garden spray kit modified to fit the Picospritzer. The pressure was adjusted to about 140 kPa (20 p.s.i.) and ejection times varied between 50 and 900 ms. The rate of ejection is strongly dependent on the tip diameter of the pipette. Many attempts had to be made to get a suitable pipette. The ejection of solution from the pipettes could be observed by the inclusion of a vital dye in the solution (normally fast green). Some experiments were performed with peptide solution containing the dye to give

an indication of the amount ejected and the area of the soma bathed in the solution. The dye did not affect the properties of the neurones. Another method used to check the pipettes was to apply a pressure pulse while observing the tip of the pipette before being placed in the recording saline. A satisfactory pipette would produce a small droplet about 20um in diameter when a 900ms pulse of pressure at 140 kPa was given. The responses obtained using pressure ejection were similar to those obtained using ionophoretic applications (Fig.2:4B).

Up to three pressure ejection pipettes could be used during one experiment. They were connected to a similar six way tap as was used for the perfusion system. The pressure output from the Picospritzer was connected to the centre position of the tap, thus the pressure was directed to one of three outflows by rotating the tap. The tap was therefore used in the opposite direction as the perfusion system (c.f. Fig.2:2).

2.6.3 BATH APPLICATION.

As only a very limited supply of the peptides was available an alternative to superfusion had to be developed. The method adopted was to inject a small amount of peptide solution into the bath with an automatic pipette (Gilson). Initially the volume of the recording chamber was 0.8ml, this had to be reduced to 0.1ml to

ensure proper mixing of the applied solution. Mixing was aided by gentle withdrawing and squirting of the saline using the Gilson pipette. During application of the peptides the recording chamber was not perfused until the response had reached a steady state.

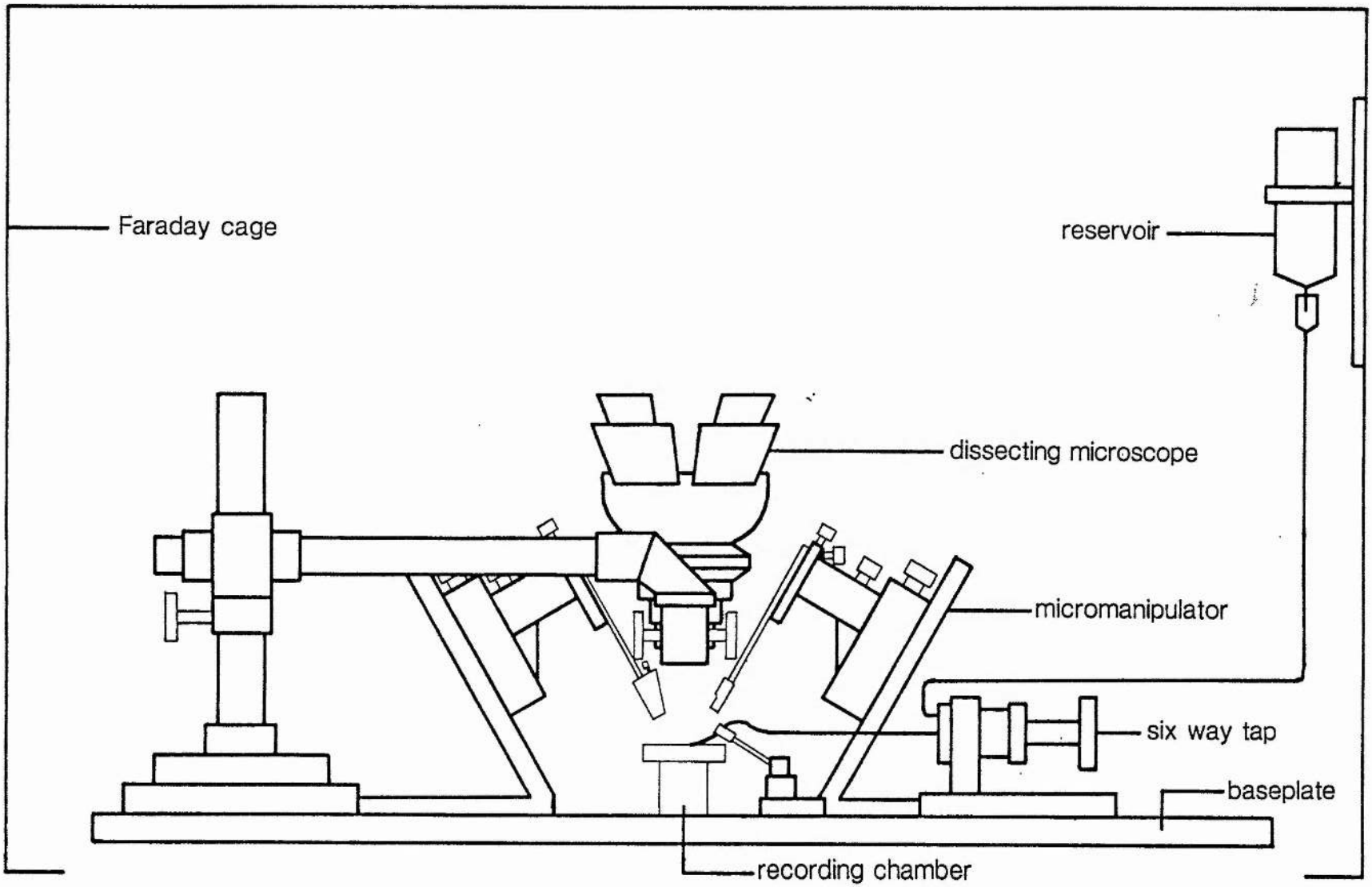
2.7 LAYOUT OF APPARATUS.

The layout of the apparatus is shown in Fig.2:5. The recording chamber, three micromanipulators, lighting system, microscope and perfusion system were all placed on a steel baseplate. The baseplate rested on foam rubber blocks on a table, which itself rested on cushioned blocks, thus reducing vibration and so aiding stable recordings.

The baseplate and the equipment mentioned above were all encased in a Faraday cage which was connected to earth to reduce electrical interference. The microscope, baseplate and one of the manipulators were also connected to earth. The earth used was that of the Tektronix oscilloscope. The electronic apparatus were mounted in a rack on the left of the cage.

Fig.2:5. Diagram showing the layout of the apparatus on the baseplate. For simplicity a third micromanipulator, light source and suction system have been omitted from this diagram. The recording electrode was connected to the micromanipulator on the right and ionophoretic and pressure ejection pipettes were connected to the left manipulator and also to the third manipulator which was placed in front of the tap.

The baseplate, Faraday cage, microscope and left manipulator were all connected to the earth of the Tektronix 502A oscilloscope.


Faraday cage
reservoir
dissecting microscope
micromanipulator
six way tap
baseplate
recording chamber

CHAPTER 3

RESULTS

3.1 PROPERTIES OF THE IDENTIFIED NEURONES INVESTIGATED.

Investigations of the actions of FMRFamide neuropeptides were made on various neurones in the cerebral, right parietal and visceral ganglia of Helix aspersa. Some of the properties of two frequently investigated neurones, the C1 and F2 neurones, are described here. Although every neurone behaves slightly differently, the behaviour of these two neurones reflect the general electrical properties of Helix neurones.

The C1 neurone, situated in each cerebral ganglion (left and right), is readily identifiable by its position (see Fig.3:1) and large size (about 200 μm in diameter). This neurone responded to FMRFamide with a combination of an increase and decrease in gK (Cottrell, 1982a; Cottrell, Davies and Green, 1984) and has been extensively studied.

Immediately following the insertion of an electrode, the C1 neurone produced action potentials, but generally after recovery from impalement the neurone became quiescent, action potentials occurring only occasionally. Recorded resting potentials of C1 neurones varied between -50 and -65 mV. A voltage (current clamp) recording obtained from a C1 neurone at its resting potential is shown in Fig.3:2. An example of an action potential recorded from a C1 neurone is shown in Fig.3:3. This particular action potential had an overshoot of about

10 mV and a duration at -30 mV of 9.2 ms.

Steady state current-voltage (I-V) relationships of neurones were obtained under voltage clamp. The holding potential was varied between -80 and -15 mV, and the net current was plotted against holding potential. The I-V curves of C1 neurones obtained using this method were non-linear, with delayed rectification occurring at membrane potentials more positive than -40 mV. An example of an I-V curve of a C1 neurone is shown in (Fig.3:4).

There are many large neurones in the suboesophageal ganglia which cannot be identified according to position and size alone (Kerkut, Lambert, Gayton, Loker and Walker, 1975). Some could, however, be identified by virtue of their electrical and pharmacological characteristics. One such neurone in the right parietal ganglion responded to FMRFamide with a combination of an increase in gNa and an increase in gK (Cottrell, Davies and Green, 1984). On the basis of its position in the ganglion, and its response to ACh and 5-HT, this neurone was identified as F2 (c.f. Kerkut et al., 1975).

Voltage recordings obtained from F2 neurones exhibited a high frequency of both action potentials and post synaptic potentials. Typical voltage recordings of the activity of C1, F1 and F2 neurones are shown in Fig.3:2. The action potentials recorded from F2 neurones

were rapid compared with the action potentials recorded from other Helix neurones, being about three times faster than those recorded from C1 and F1 neurones (Fig.3:3).

The I-V relationship of an F2 neurone is shown in Fig.3:5. Delayed rectification, similar to that observed in the C1 neurone, occurred at membrane potentials more positive than -40 mV. However, anomalous rectification was also evident in F2 neurones at hyperpolarized potentials. This increase in membrane conductance with hyperpolarization was also detected as a decrease in the amplitude of electrotonic potentials resulting from hyperpolarizing current pulses. The electrical properties mentioned above, particularly the synaptic activity and spike duration, were used to identify the F2 neurone from preparation to preparation.

Fig.3:1. Diagram showing the localization of the C1 neurone in each cerebral ganglion. This neurone could be identified solely by its size and postion on the ventral side of the cerebral ganglia.

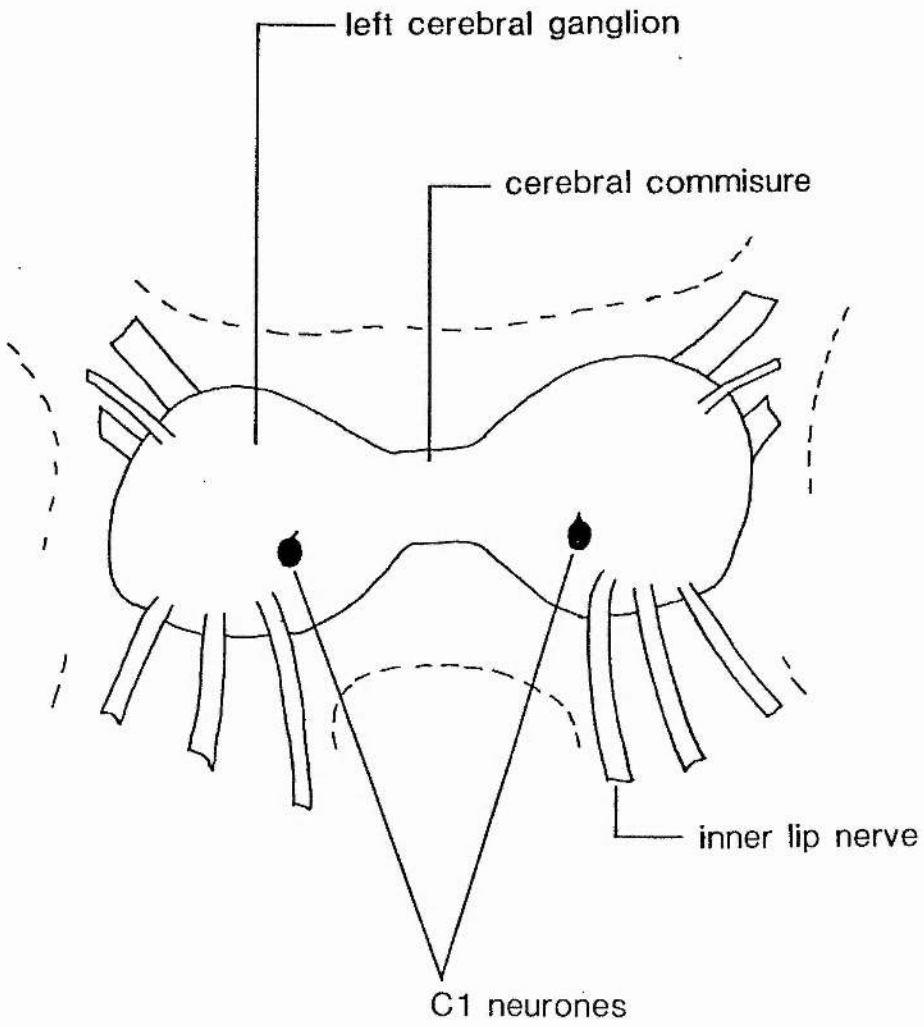

left cerebral ganglion
cerebral commisure
inner lip nerve
C1 neurones

Fig.3:2. Voltage recordings of the electrical activity observed in C1, F2 and F1 neurones. The C1 neurone fired an action potential immediately following impalement (arrow) and then became quiescent. The F2 neurone displayed had a large number of post synaptic potentials and frequently occurring action potentials. The F1 neurone exhibited a bursting pattern which was endogenous and characteristic of the F1.

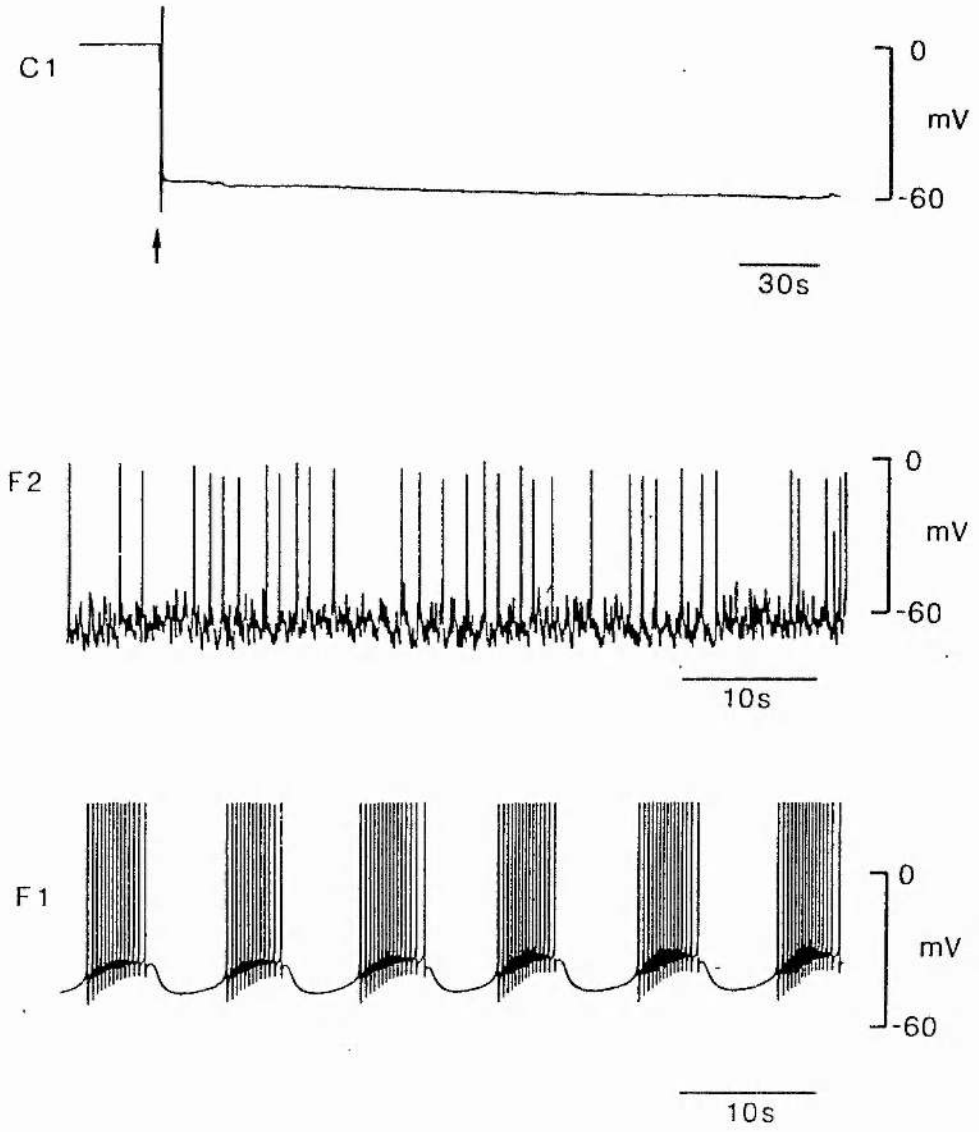

C1
0
mV
-60
30s
F2
0
mV
-60
10s
F1
0
mV
-60
10s

Fig.3:3. Comparison of the action potentials recorded from the C1, F1 and F2 neurones. The duration of the action potentials at -30 mV were 9.2 and 11.2 ms for the C1 and F1 neurones respectively while for the F2 neurone the duration was much shorter, being only 3 ms.

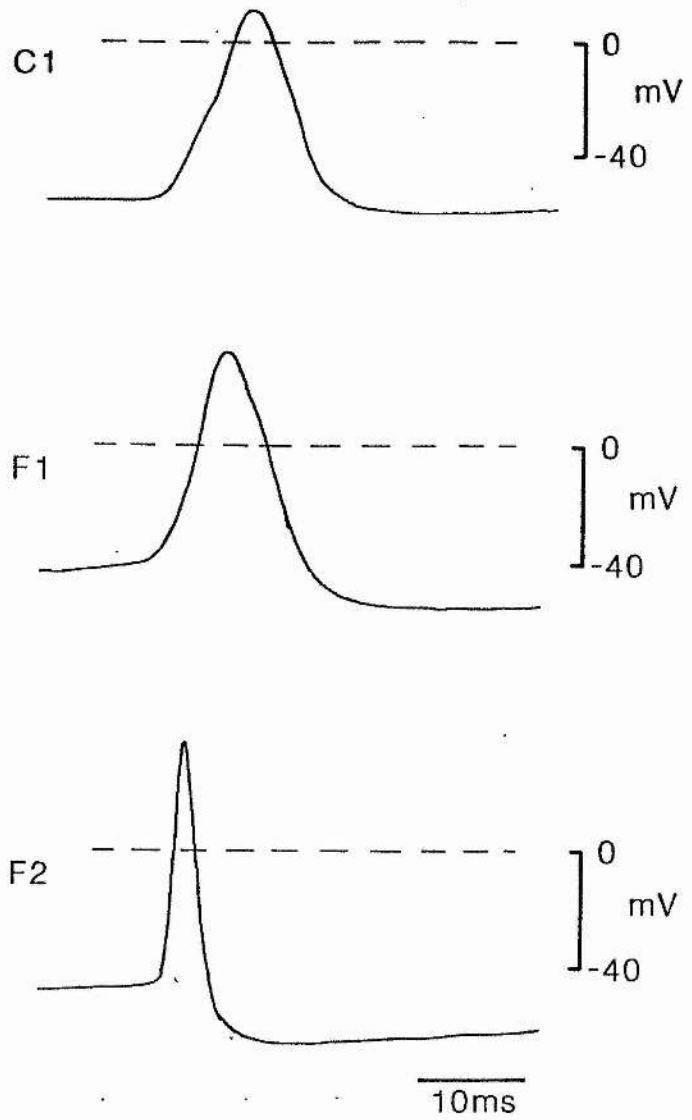

C1
0
mV
-40
F1
0
mV
-40
F2
0
mV
-40
10ms

Fig.3:4. Current-voltage (I-V) relationship of a C1 neurone. Delayed rectification began to occur at -30 mV. Vh, holding potential; Iss, steady state current.

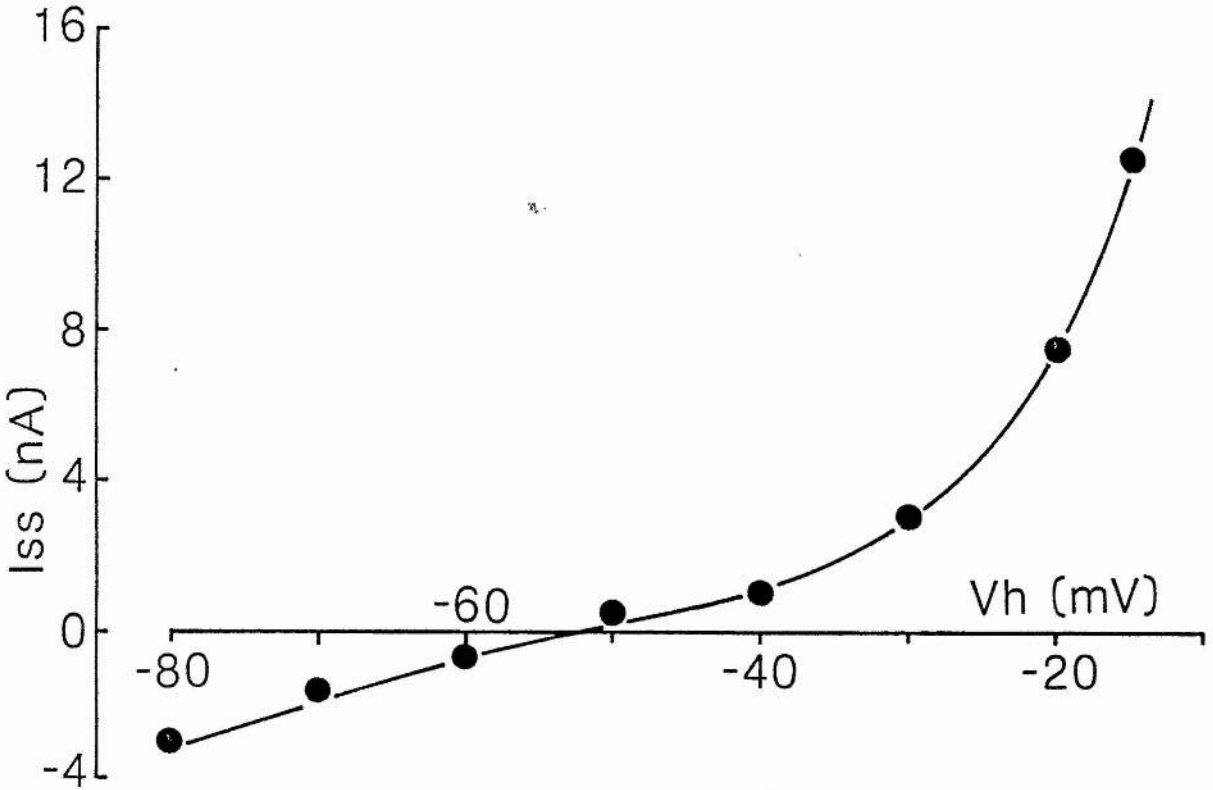
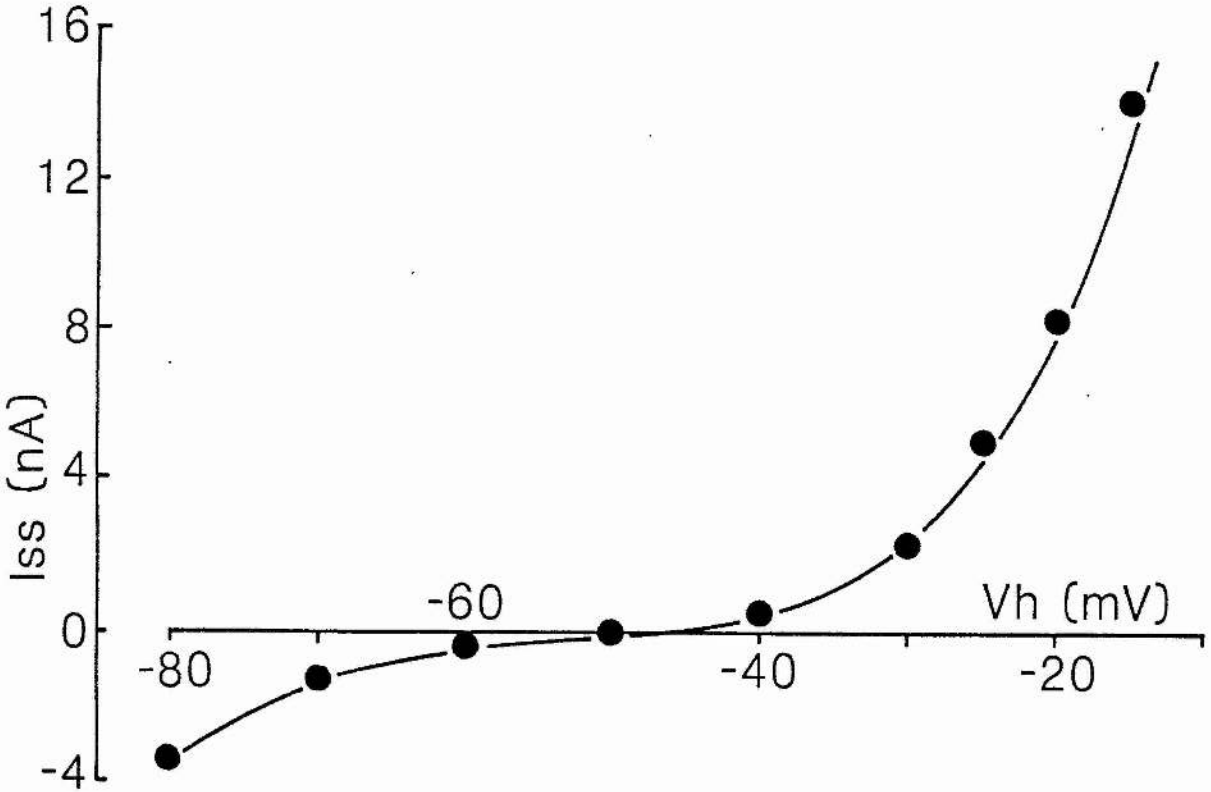

16
12
8
4
0
-4
Iss (nA)
-80
-60
-40
-20
Vh (mV)

Fig.3:5. I-V relationship of an F2 neurone. As with the I-V curve of the C1 neurone, delayed rectification began to occur at around -30 mV (c.f. Fig.3:4.). In addition to delayed rectification this neurone also displayed anomalous rectification. This is an increase in membrane conductance resulting from hyperpolarization which is seen as an increase in the slope of the I-V curve at membrane potentials more negative than -60 mV. Vh, holding potential; Iss, steady state current.


16
12
8
4
0
-4
Iss (nA)
-80
-60
-40
-20
Vh (mV)

3.2 THE EFFECT OF FMRFamide ON IONIC CONDUCTANCES.

The effects of FMRFamide on Helix neurones were investigated by ionophoretic application of the peptide onto the soma of individual neurones. Some of the actions of FMRFamide, particularly on the C1 neurone, have been investigated previously by Cottrell (1978, 1979, 1982a). I have confirmed many of the results published by Cottrell, and also undertaken a more detailed investigation of the ionic mechanisms involved in producing the FMRFamide responses.

Three different responses to FMRFamide have been observed:

(1) increase in gK

(2) increase in gNa

(3) decrease in gK

(Cottrell, Davies and Green, 1984). Recently Boyd and Walker (1985) have reported depolarizing and hyperpolarizing responses to FMRFamide in neurones of the suboesophageal ganglia of Helix. Many neurones responded with a combination of two or more of the above responses, in some instances making the analysis of the ionic mechanisms complicated. This was especially true of the C1 neurone which responded to FMRFamide with a combination

of an increase in gK and a voltage-dependent decrease in gK (Cottrell, 1982a). The combination of responses occurring on single neurones has implications regarding multiple receptor sites for FMRFamide and related peptides on Helix neurones. This will be discussed in detail later.

Most of the experiments reported here were repeated at least 3 or 4 times. However, in some cases the number of experiments performed was limited. In such cases the number of the experiments conducted (n) will be indicated.

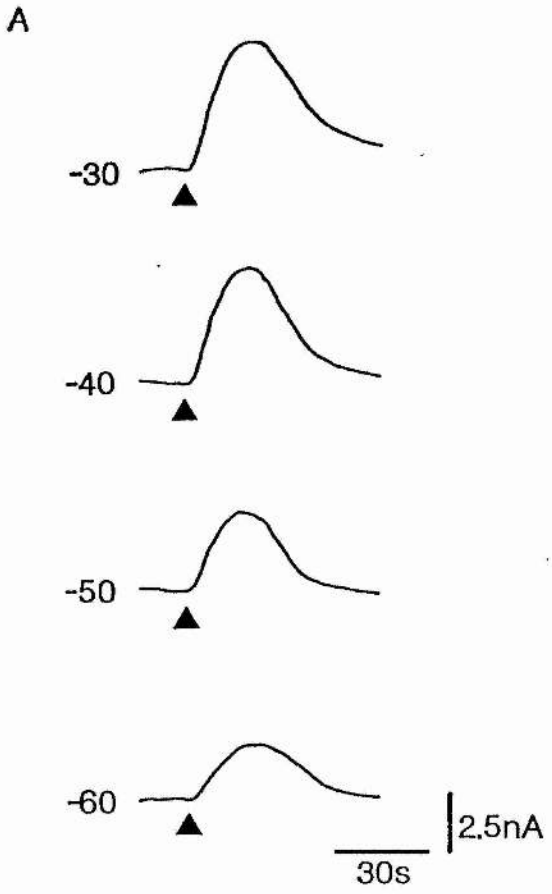
3.3 THE HYPERPOLARIZING RESPONSE.

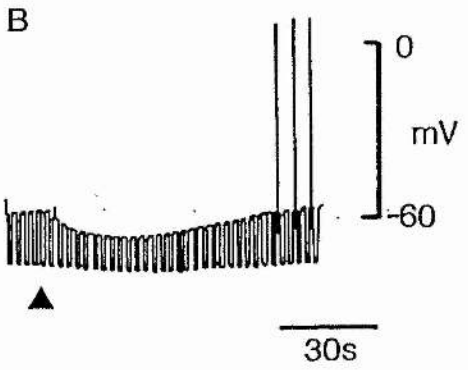
3.3.1 PROPERTIES OF THE HYPERPOLARIZATION INDUCED BY FMRFamide.

In addition to the C1 neurone in each cerebral ganglion, many neurones in the suboesophageal ganglia were hyperpolarized by FMRFamide. In the C1 neurone, FMRFamide induced a voltage dependent inward current at membrane potentials more positive than -40 mV (Cottrell, 1982a). However, in many neurones of the suboesophageal ganglion, FMRFamide appeared to activate a hyperpolarizing response alone. During voltage clamp, the response was detected as an outward current (see Fig.3:6).

The relationship between the FMRFamide induced current and membrane potential was obtained by applying FMRFamide while the neurone was voltage clamped at various holding potentials. The non-linearity of the relationship between the FMRFamide induced current and the holding potential is shown in Fig.3:7. When the holding potential was more negative than -75 mV, the outward current was reversed to an inward current.

Fig.3:6. A, application of FMRFamide (▲) onto a C1 neurone during voltage clamp induced an outward current at all potentials shown here. The holding potential (mV) is indicated by the side of each record. B, the hyperpolarizing response recorded during current clamp. The amplitude of electrotonic potentials resulting from the application of constant amplitude hyperpolarizing pulses was reduced during the response, suggesting that the conductance of the neurone was increased. C, in a C1 neurone voltage clamped at -25 mV, the amplitude of current deflections resulting from 15 mV hyperpolarizing command pulses was increased during the response, again suggesting an increase in conductance.


A
-30
-40
-50
-60
2.5nA
30s


B
0
mV
-60
30s

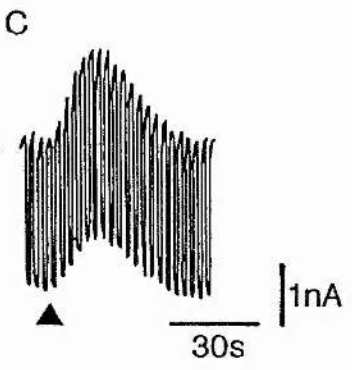
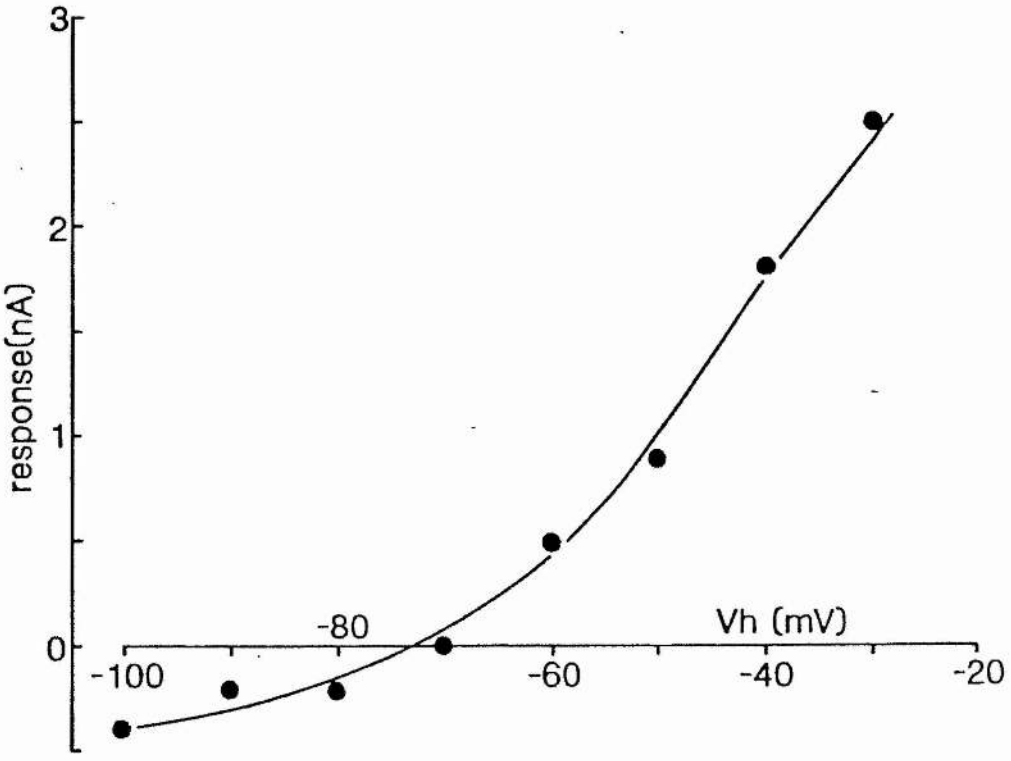

C
1nA
30s

Fig.3:7. The relationship between holding potential and the amplitude of the FMRFamide induced current in a voltage clamped C1 neurone. The response reverses between -70 and -80 mV, which is close to E_K in snail neurones. Vh, holding potential.


3
2
1
0
response(nA)
-80
Vh (mV)
-100
-60
-40
-20

3.3.2 IONIC MECHANISM OF THE HYPERPOLARIZING RESPONSE.

Hyperpolarizing responses may occur either by a reduction in a steady state inward current, resulting from a decrease in conductance, or by the generation of an outward current, resulting from an increase in conductance. An indication of the change in conductance occurring in a neurone may be obtained by passing constant amplitude current pulses into the cell at regular intervals. Provided the pulses are long enough to fully charge the capacitance of the membrane, the resulting voltage deflections are proportional to the resistive conductance of the membrane. The method during voltage clamp is to apply regular voltage command steps while monitoring the amplitude of the resulting current deflections.

The amplitude of the voltage deflections produced by injecting constant amplitude hyperpolarizing current pulses was decreased during the application of FMRFamide (Fig.3:6). The effect was also observed as an increase in the amplitude of current deflections resulting from command pulses during voltage clamp. These observations strongly suggest that the hyperpolarizing response is accompanied by an increase in conductance. However, care must be taken in interpreting such observations as pointed out by Dingledine (1983), see also page 124.

For a hyperpolarizing response resulting from an increase in conductance to occur, the ionic species involved must have a combined equilibrium potential which is more negative than the resting potential of the cell. Two ionic species with an equilibrium potential negative to the resting potential are Cl^- and K^+ ions. The response is unlikely to involve an increase in gCl since it was unaffected by a 75% reduction in extracellular Cl^- concentration (Cottrell, Davies and Green, 1984).

The reversal potential of the response was close to the K equilibrium potential (E_K) of Helix neurones. Furthermore, when the extracellular K concentration was altered, there was a concomitant shift in the reversal potential of the response. The effect of reducing the external K concentration from 5 to 1 mM on the amplitude and direction of the FMRFamide induced current in a voltage clamped C1 neurone is shown in Fig.3:8. Reducing the external K concentration causes a shift of E_K in the negative direction. The change expected, as calculated using the Nernst equation, is -40.6 mV. If the hyperpolarization elicited by FMRFamide was due to a pure increase in gK, then the reversal potential of the response should follow E_K exactly. The observed change in the reversal potential of the FMRFamide response was -40 mV (see Fig.3:8). This close agreement with E_K suggested that the response was due to an increase in

conductance, mainly if not exclusively to K^+ ions.

3.3.3 EFFECT OF K CHANNEL BLOCKERS.

A number of compounds block K currents in molluscan nerve cells (Adams, Smith and Thompson, 1980). Among these, tetraethylammonium (TEA) has been shown to block delayed outward K currents in Aplysia (Hermann and Gorman, 1981b) and Tritonia (Thompson, 1977), and both delayed and Ca activated K currents in Helix (Meech and Standen, 1975).

Exposure of the preparation to 30 mM TEA reduced the amplitude of the FMRFamide response. TEA was substituted for NaCl as shown in the methods section. Although neither Na^+ ions nor Cl^- ions are involved in the response, control currents were obtained using solutions in which a corresponding amount of NaCl was substituted with sucrose. The attenuation of the response of a neurone in the right parietal ganglion during the presence of 30 mM TEA is shown in Fig.3:9. The concentration of TEA used is similar to the concentration reported to block time and voltage dependent K currents in molluscan neurones, for example Meech and Standen (1975) used 100 mM to block both delayed and Ca activated K currents in Helix, and Hermann and Gorman (1981b) used 50 mM TEA to block the delayed K current in Aplysia.

Fig.3:8. The relationship between response amplitude and holding potential following ionophoresis of FMRFamide onto a voltage clamped C1 neurone. Reducing the external K concentration from 5 (●) to 1 mM (■) resulted in a concomitant change in the reversal potential from -70 to -110 mV.

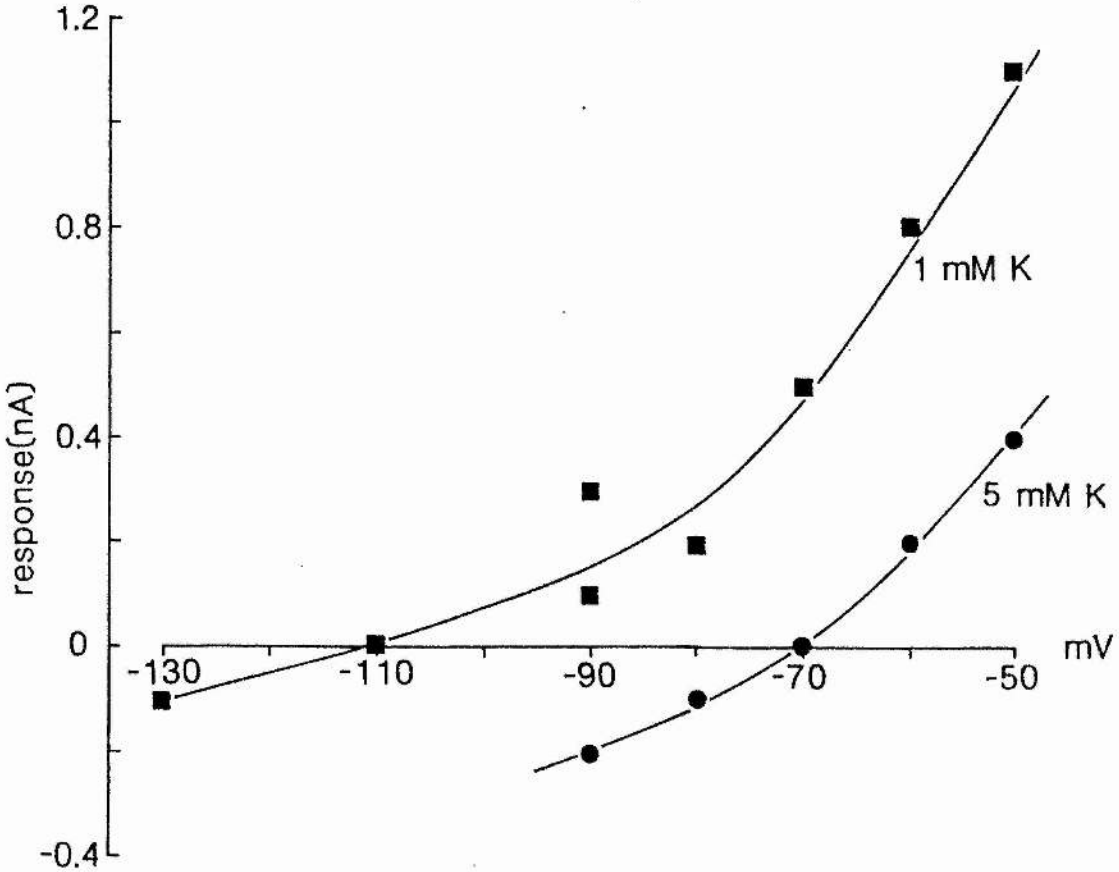
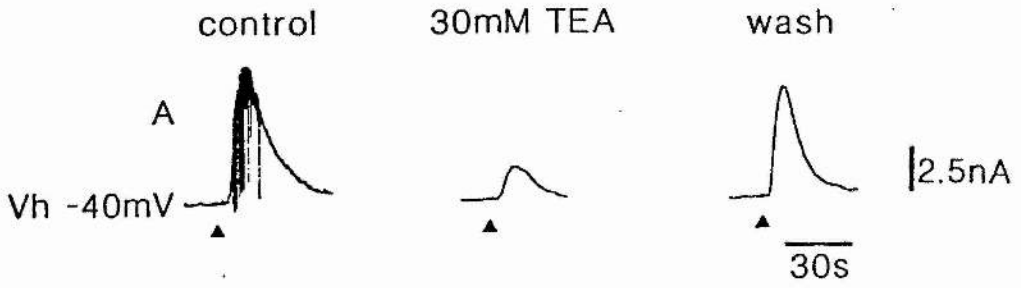
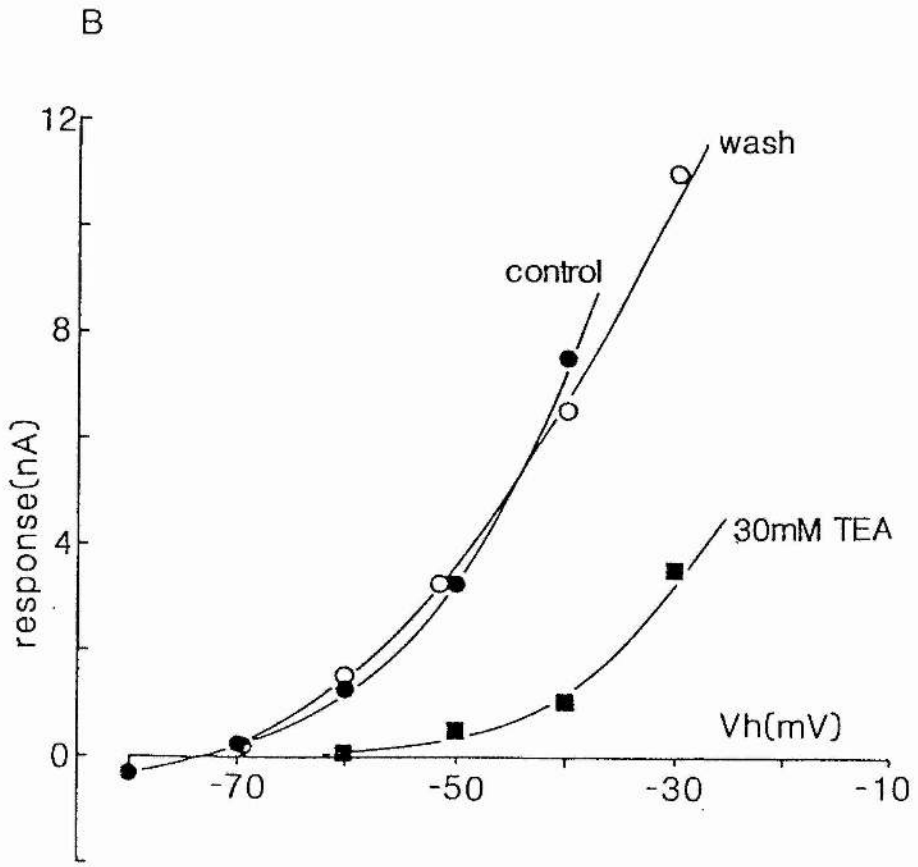

1.2
0.8
0.4
0
-0.4
response(nA)
-130
-110
-90
-70
-50
mV
1 mM K
5 mM K

Fig.3:9. The effect of TEA on the K response of a neurone in the right parietal ganglion. The presence of 30 mM TEA in the bathing solution led to a marked reduction in the amplitude of the FMRFamide induced response. A, outward currents observed to ionophoretic application of FMRFamide (▲) while the neurone was voltage clamped at -40 mV. The downward deflections in the control trace were probably unclamped axon spikes. B, relationship between membrane potential and the amplitude of the FMRFamide induced current in the presence and absence of TEA. 30 mM TEA was substituted for the corresponding amount of NaCl (see text). Vh, holding potential.


control
30mM TEA
wash
A
Vh -40mV
2.5nA
30s


B
12
8
4
0
response(nA)
wash
control
30mM TEA
Vh(mV)
-70
-50
-30
-10

Intracellular Cs^+ ions have been employed to block K currents of both Helix (Akaike, Lee and Brown, 1978) and Aplysia neurones (Tillotson and Horn, 1978). In this study, Cs^+ ions were injected intracellularly using recording electrodes filled with 2M CsCl. After impalement, the injection of Cs into the cell was facilitated by passing a positive current through the electrode. Generally the effect of Cs was observed after 2 to 4 minutes. This was evident as a decrease in outward current required to voltage clamp the cell at depolarized potentials. The I-V curve of a neurone, obtained 15 minutes following impalement with a CsCl electrode, is shown in Fig.3:10. The effect of intracellular Cs on the FMRFamide induced K current was investigated by impaling a C1 neurone with a CsCl electrode, voltage clamping the neurone at -40 mV, then applying FMRFamide at regular intervals (Fig.3:11). The FMRFamide response rapidly declined with time, and after 7 minutes it was virtually abolished. There was also an accompanying decrease in the steady state K currents. In Aplysia, a slow cholinergic IPSP resulting from an increase in gK is also blocked by intracellular Cs (Kehoe, 1972a).

Fig.3:10. I-V relation of a C1 neurone during the presence of intracellular Cs^+ ions. A C1 neurone was impaled with a CsCl recording electrode and left for about 15 minutes to allow Cs^+ ions to enter the cell. Although using this method of introducing Cs into the cell makes it impossible to obtain a control I-V curve, that of Fig.3:4. is shown here for comparison (dashed line). Outward K currents were markedly reduced by the Cs.

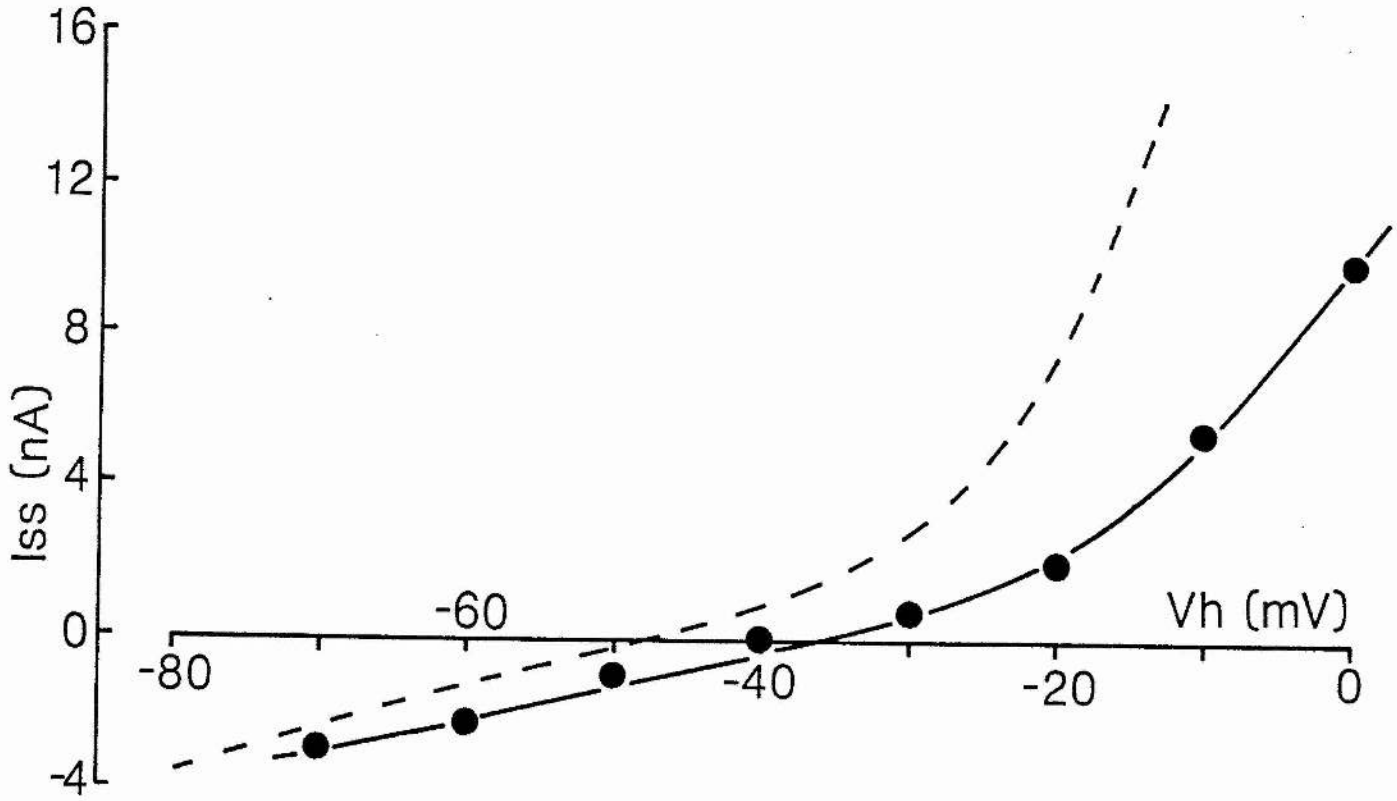
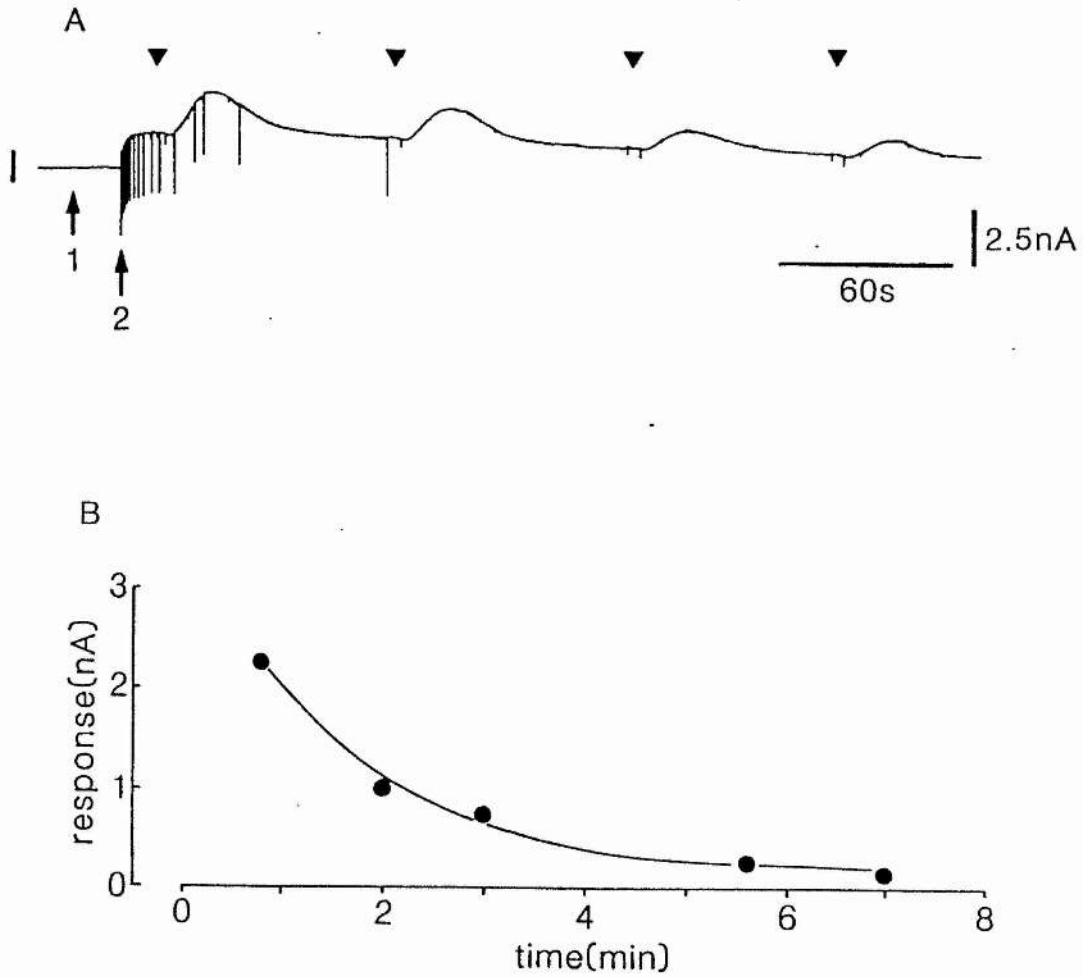

16
12
8
4
0
-4
Iss (nA)
-80
-60
-40
-20
0
Vh (mV)

Fig.3:11. The effect of intracellular Cs on the K response induced by FMRFamide. A, current trace obtained from a C1 neurone voltage clamped at -40 mV. At point 1 the cell was impaled with a CsCl filled recording electrode and at point 2 the cell was voltage clamped. Ionophoretic application of FMRFamide (▼) produced outward K current responses which rapidly diminished with time. B, the amplitude of the current responses of a different experiment plotted against time after impalement. The response was virtually abolished after 7 minutes.

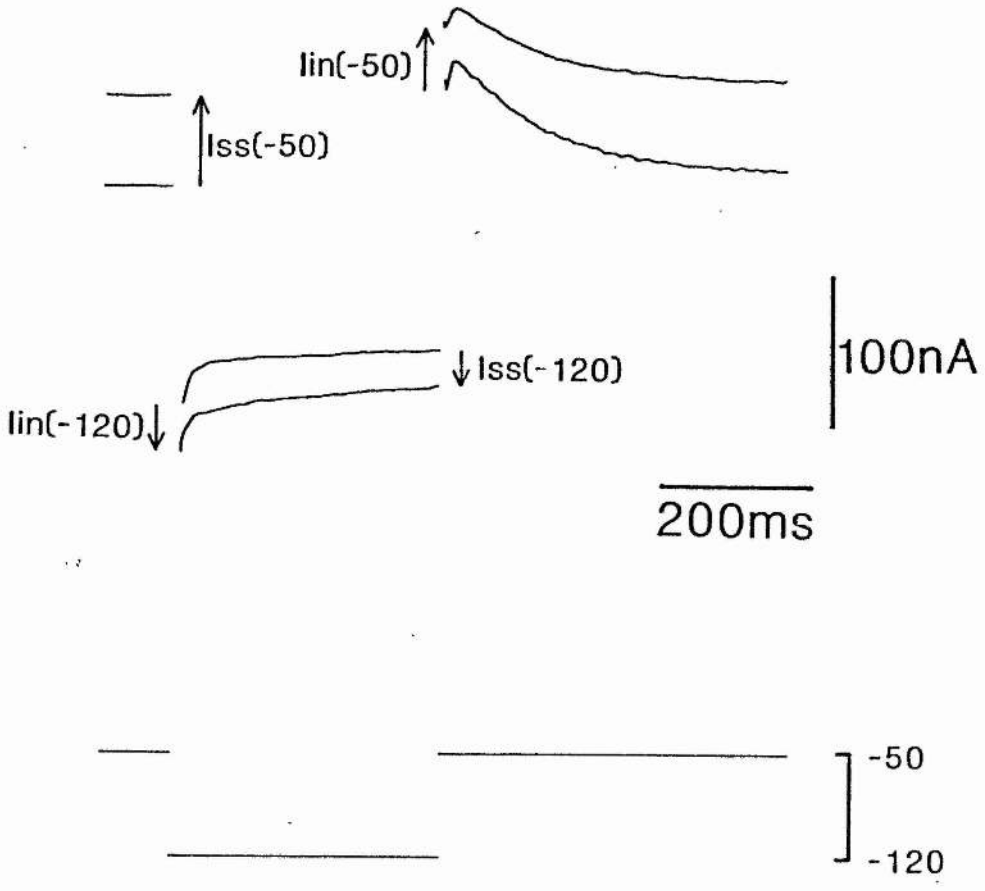

A
1
2
2.5nA
60s
B
response(nA)
0
1
2
3
0
2
4
6
8
time(min)

3.3.4 RELAXATION EXPERIMENTS ON THE HYPERPOLARIZING RESPONSE.

Relaxation or voltage jump experiments were performed using a voltage clamp amplifier based on the design of Lee, Akaike and Brown (1980). This amplifier could voltage clamp a neurone much faster than the switched single electrode clamp system, and could be used in both single and double electrode clamp mode.

Relaxation experiments have provided valuable information about the mechanisms of neurotransmitter actions (e.g. Neher and Sackman, 1975; Marty and Ascher, 1978; Adams and Brown, 1980). A relaxation experiment involved comparing the currents elicited by a command potential in the presence and absence of FMRFamide. The currents obtained when a C1 neurone was voltage clamped at -50 mV, and a command pulse to -120 mV applied is shown in Fig.3:12. The control currents and the currents observed in the presence of FMRFamide were superimposed on a storage oscilloscope. The FMRFamide was applied by injecting 20 µl of a 1 mM solution of FMRFamide to the recording chamber. FMRFamide produced an outward current at -50 mV, which was reversed to an inward current at -120 mV (n=1). The FMRFamide induced current was obtained by subtracting the control current from the current in the the presence of FMRFamide. The FMRFamide induced

Fig.3:12. An example of a relaxation experiment conducted on a C1 neurone. The cell was voltage clamped at -50 mV and a command potential to -120 mV applied, first in the absence, and then in the presence of FMRFamide. Both traces are superimposed here. The arrows designate the direction and approximate amplitude of the FMRFamide induced currents. Iin(-50) and Iin(-120), instantaneous currents; Iss(-50) and Iss(-120), steady state currents.


Iin(-50)
Iss(-50)
Iss(-120)
Iin(-120)
100nA
200ms
-50
-120

conductance was calculated from:

$$g_{Fa} = I_{Fa}/(V - E_K) \qquad (3.1)$$

where g_{Fa} and I_{Fa} are the FMRFamide induced conductance and current respectively. A value of -80 mV was assumed for E_K, and V had the values of -50 and -120 mV. The FMRFamide induced conductance just before, during, and after the command step is shown in Fig.3:13. Immediately following the step from -50 to -120 mV the conductance fell to a new value which reflected the rectification occurring in the FMRFamide activated channels. The assumption made in applying this argument is that there is no instantaneous change in the number of FMRFamide activated channels which are open. The decrease of the conductance occurring during the step is the relaxation of the conductance to its new steady state value at -120 mV. On stepping back to -50 mV there is again an instantaneous change in the conductance, which subsequently increased to its steady state value at -50 mV. The ratio of the single channel conductance (γ) at -120 mV to that at -50 mV is given by:

$$\gamma(-120)/\gamma(-50) = g_{ss}(-120)/g_{in}(-50) \qquad (3.2)$$

where g_{in} and g_{ss} are the instantaneous and steady state conductances respectively. This ratio, which reflects the rectification of the channels as mentioned above, was

0.46.

The relaxation of the FMRFamide induced conductance to a new value following a voltage jump implied that the number of channels open at any one time is a function of voltage. The steady state FMRFamide induced conductance (g_{ss}) at a potential V is given by:

$$g_{ss}(V) = N_{ss}(V) . \gamma(V) \qquad (3.3)$$

where $N_{ss}(V)$ is the steady state number of open channels at potential V, and $\gamma(V)$ is the single channel conductance at potential V. Dividing this equation for V = -120 with that for V=-50 gives:

$$\frac{g_{ss}(-120)}{g_{ss}(-50)} = \frac{N_{ss}(-120)}{N_{ss}(-50)} \frac{\gamma(-120)}{\gamma(-50)} \qquad (3.4)$$

Combining equations (3.2) and (3.4) and rearranging gives:

$$N_{ss}(-120)/N_{ss}(-50) = g_{in}(-50)/g_{ss}(-50) \qquad (3.5)$$

which, upon inserting the appropriate values, gave:

$$N_{ss}(-120)/N_{ss}(-50) = 0.6$$

Therefore, the steady state number of FMRFamide activated

channels open at -120 mV is only 60% of the number open at -50 mV.

A semi-logarithmic plot of the relaxation after stepping from -120 to -50 mV was linear, implying that the relaxation followed a single exponential, which had a time constant of 136 ms (Fig.3:14). It is interesting to note that the decrease in conductance following a hyperpolarizing step contrasts with the situation occurring during the ACh induced K current in Aplysia, where the conductance following a hyperpolarizing step increases with time (Marty and Ascher, 1978).

Fig.3:13: Plot of the conductance values calculated from the experiment shown in Fig.3:12. The conductance was calculated using a value of -80 mV for the K equilibrium potential and using the equation:

$$g_{Fa} = I_{Fa}/(V - E_K)$$

where g_{Fa} is the FMRFamide conductance and I_{Fa} is the FMRFamide induced current.

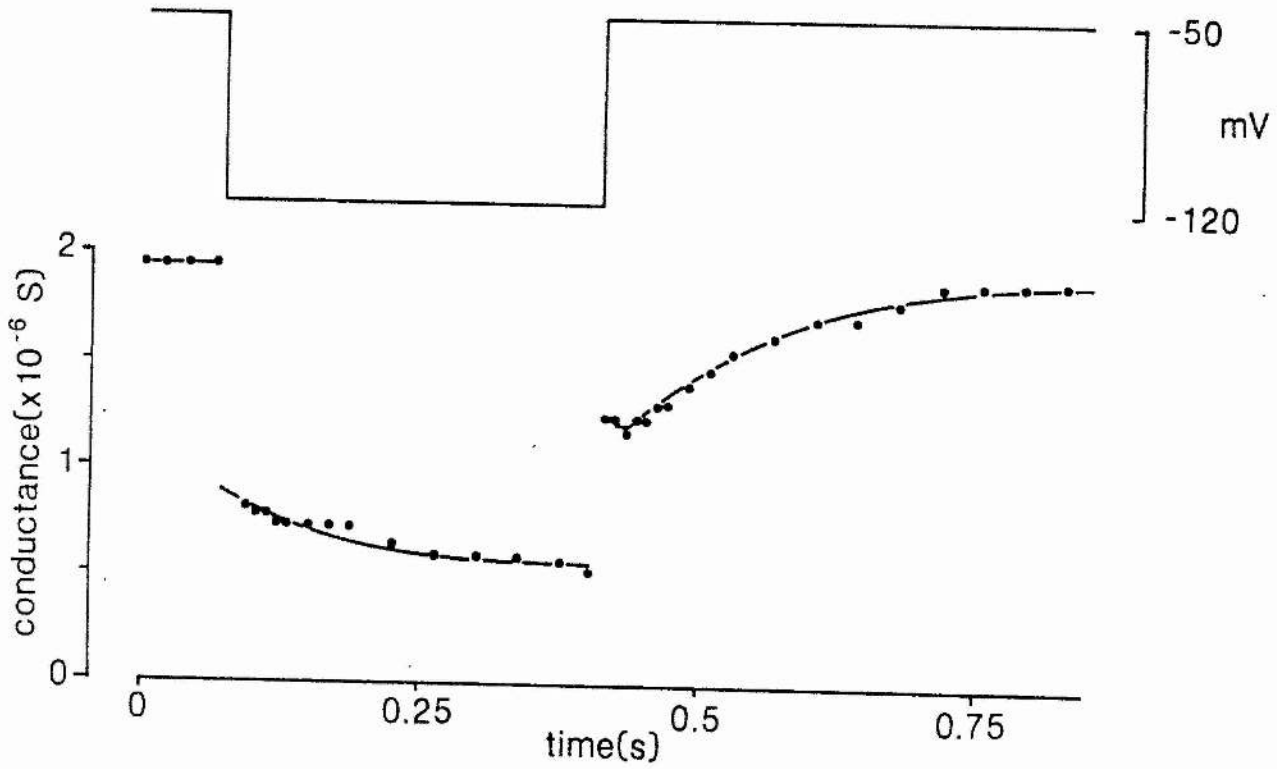
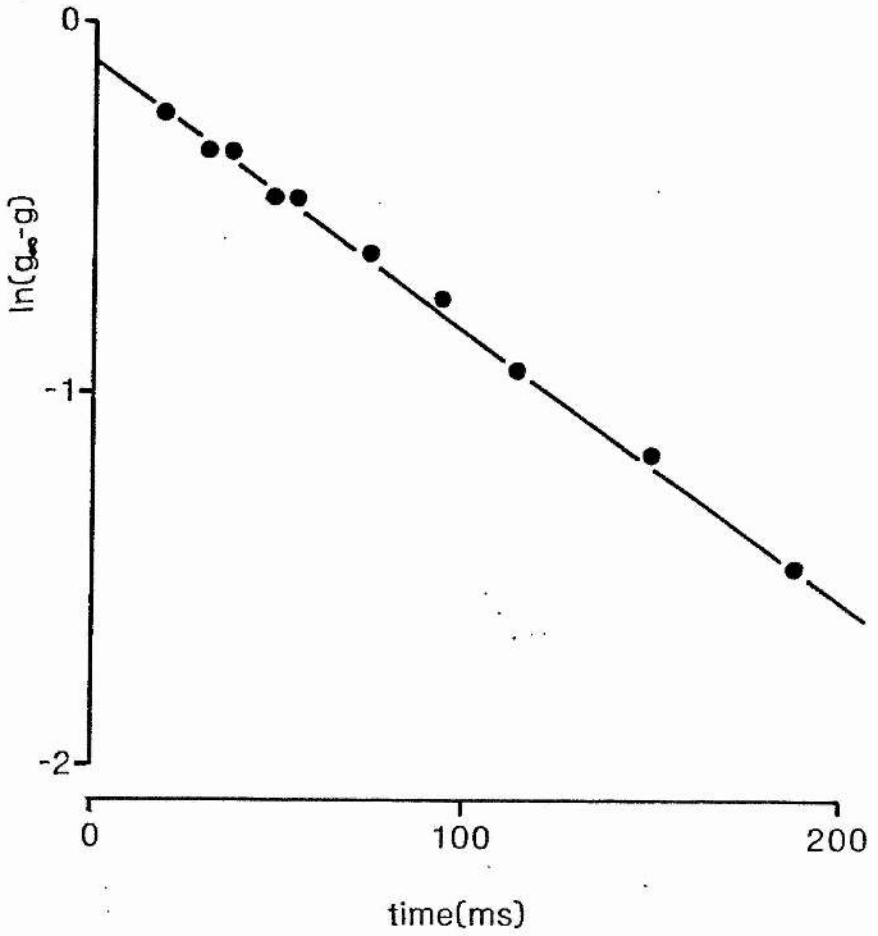

-50
mV
-120
2
1
0
conductance(x10^{-6} S)
0
0.25
0.5
0.75
time(s)

Fig.3:14. Semi-logarithmic plot of the increase in the FMRFamide induced conductance following the step back from -120 to -50 mV. The data is that of Fig.3:13. A value of 1.96×10^{-6} S was given to g_{∞}, which is the steady state conductance induced by FMRFamide at a holding potential of -50 mV. The relaxation of the conductance to its steady state value appears exponential, with a time constant of 136 ms.


0
-1
-2
ln(g∞-g)
0
100
200
time(ms)

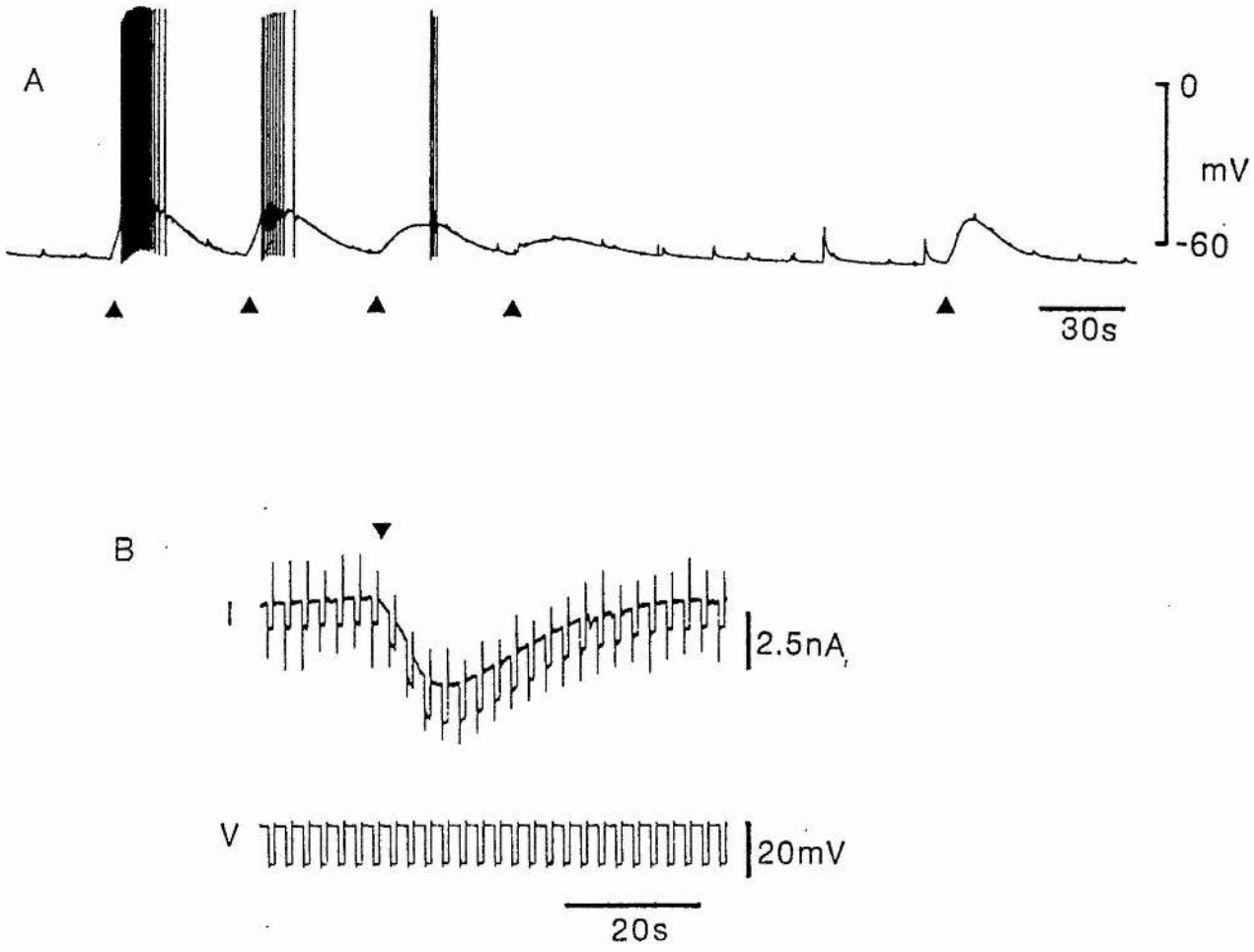
3.4 THE DEPOLARIZING RESPONSE.

3.4.1 PROPERTIES OF THE DEPOLARIZATION INDUCED BY FMRFamide.

Not as many neurones were depolarized as were hyperpolarized by FMRFamide. Furthermore, two neurones which gave a depolarizing response to FMRFamide were subsequently found to respond with a combination of two different conductance mechanisms. These were the C2 neurone in each cerebral ganglion and the F2 neurone in the right parietal ganglion, both of which gave a mixed response involving an increase in gNa and gK. However, a neurone in the vicinity of E13 in the visceral ganglion appeared to give a pure depolarizing response to FMRFamide.

In contrast to the hyperpolarization, repeated application of FMRFamide led to a rapid desensitization of the depolarizing response (Fig.3:15). Under voltage clamp conditions, the response was detected as an inward current. The relationship between response amplitude and holding potential was linear over the range investigated (Fig.3:16A). The amplitude of the current elicited by FMRFamide increased as the cell was hyperpolarized and decreased as the cell was depolarized, although reversal of the response was not observed.

Fig.3:15. The depolarizing response to FMRFamide recorded from a visceral ganglion neurone. A, voltage recording showing the rapid desensitization of the response by repeated application of FMRFamide (▲). The response recovered after a short period in the absence of peptide. B, the response was detected as an inward current when the neurone was voltage clamped at -50 mV. During the recording, 15 mV hyperpolarizing command pulses were continuously applied to the cell. The amplitude of the resulting current deflections was increased during the response, suggesting that the membrane conductance was increased.

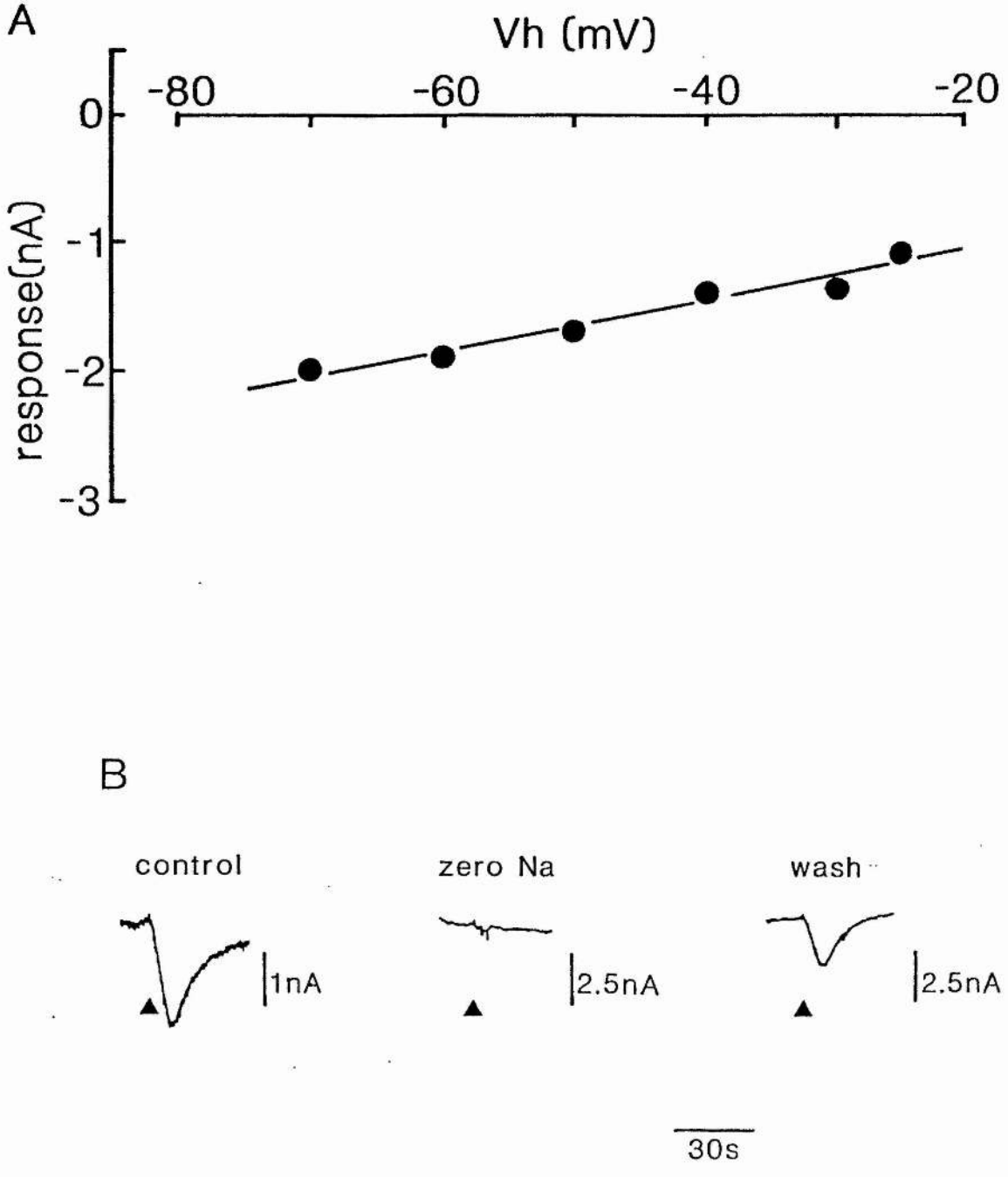

A
0
mV
-60
30s
B
I
2.5nA
V
20mV
20s

3.4.2 IONIC MECHANISM OF THE DEPOLARIZING RESPONSE.

As with the hyperpolarization, the amplitude of current deflections resulting from regular command pulses during voltage clamp was increased during the response. This implied that the depolarizing response was also associated with an increase in membrane conductance. By the same argument as was used for the hyperpolarization, a depolarizing response associated with an increase in conductance must involve an ionic species with an equilibrium potential which is positive to the resting potential. Two ions which satisfy this requirement are Na^+ and Ca^{2+} ions.

If the depolarization involved an increase in gNa, then reducing the extracellular Na concentration would lead to an attenuation in the amplitude of the response. Exposure of the preparation to zero Na solution completely abolished the current induced by FMRFamide application. This effect was observed over a range of holding potentials from -70 to -30 mV (Fig.3:16B). The abolition of the response in zero Na suggests that Na^+ ions are the predominant charge carriers producing the depolarization.

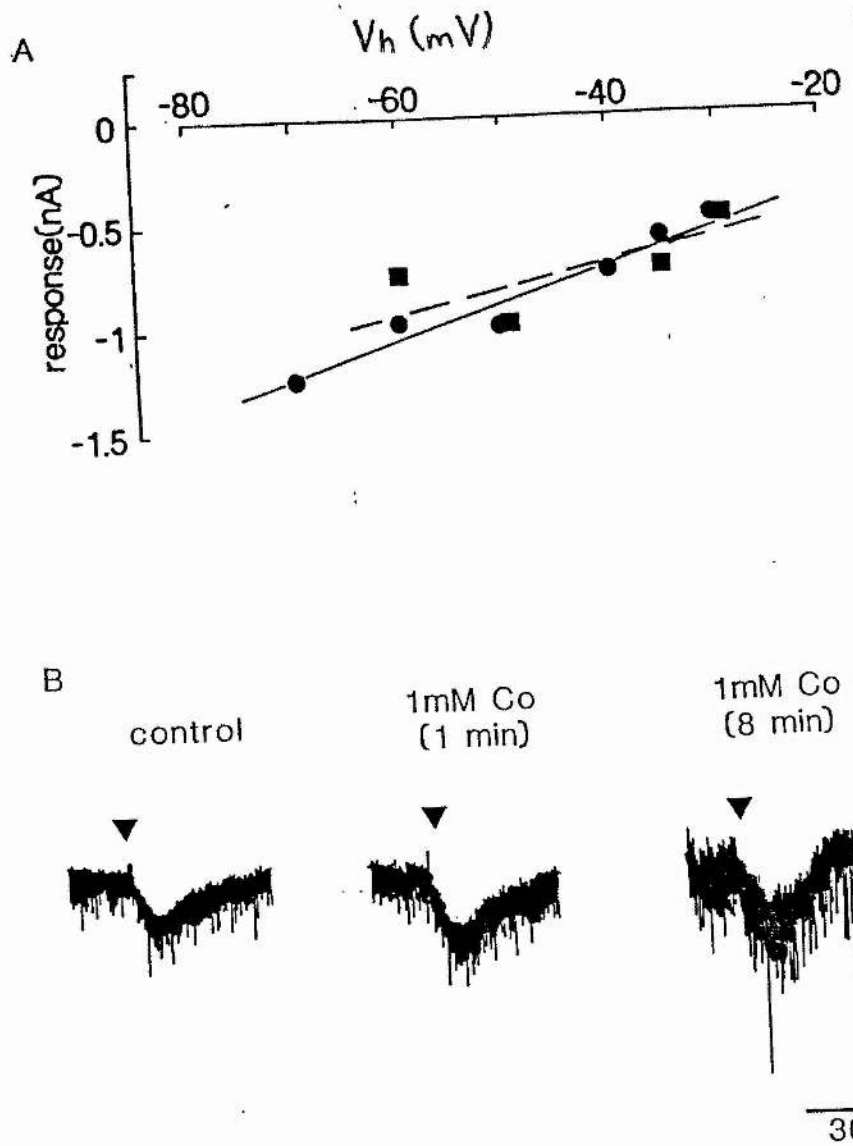
Fig.3:16. A, plot of the amplitude of the FMRFamide induced inward current in a visceral ganglion neurone voltage clamped at various holding potentials. B, effect of zero Na on this response. Exposure of the preparation to zero Na (NaCl replaced with sucrose) completely abolished the response, which recovered after a time in normal solution. Note the difference in scale, holding potential was -40 mV.


A
Vh (mV)
-80
-60
-40
-20
0
-1
-2
-3
response(nA)
B
control
zero Na
wash
1nA
2.5nA
2.5nA
30s

3.4.3 EFFECT OF CHANNEL BLOCKERS.

As shown previously, 30 mM TEA markedly reduced the FMRFamide induced K current. This concentration of TEA appeared to have no effect on the FMRFamide induced inward Na current recorded from a neurone in the vicinity of E13 in the visceral ganglion (Fig.3:17). This result implied that the response to FMRFamide in this neurone was not contaminated by the increase in gK response to FMRFamide. However, it does not rule out the possibility that K^+ ions cross the membrane during this response. For example, the depolarization induced by ACh in Helix pomatia neurones consists of an increase in permeability to Na^+, K^+ and Ca^{2+} ions in the ratio 1 : 1.6 : 0.1 (Witte, Speckmann and Walden, 1985). Ascher, Marty and Nield (1978) have reported similar conductance mechanisms occurring in Aplysia neurones depolarized by ACh. These ions are believed to pass through a fairly large channel, permeable to all three ions. If the FMRFamide induced depolarization involved the activation of a similar cation selective channel, then TEA, and other K channel blockers such as Cs^+ ions (see below) would not discriminate against K^+ ions passing through this type of channel.

Fig.3:17. A, the effect of TEA on the depolarizing response recorded under voltage clamp. Exposure of the preparation to 30 mM TEA did not affect the amplitude of the FMRFamide induced inward current over a range of holding potentials between -70 and -30 mV. TEA was substituted for a corresponding amount of NaCl. (●) control, (■) 30 mM TEA. The solid line was fitted by eye through the control points and the broken line was fitted by eye through the TEA points. B, influence of 1 mM Co on the depolarizing response recorded from an F2 neurone. There was no apparent decrease in the amplitude of the response after 8 minutes in Co solution. These current records were obtained at a holding potential of -70 mV. There is extensive synaptic activity present on these records. The recording electrode was filled with CsCl (see later in text).


A
Vh (mV)
-80
-60
-40
-20
0
-0.5
-1
-1.5
response(nA)
B
control
1mM Co
(1 min)
1mM Co
(8 min)
1nA
30s

N-methyl aspartate activates a voltage sensitive Ca current in rat hippocampal neurones (Dingledine, 1983). This agonist activated Ca current is blocked by conventional Ca channel blockers such as Co. The Ca current of Helix neurones is also blocked by Co at concentrations of 1 mM (Akaike, Lee and Brown, 1978; Barnes, Cottrell and Dunbar, 1985). The depolarizing response to FMRFamide was unaffected by 1 mM Co (n=2, Fig.3:17), although the same argument applies here as for the case of TEA described above.

The effect of intracellular Cs on the FMRFamide induced inward current was investigated using a similar protocol as for the hyperpolarizing response. Despite a reduction in the outward K currents, the FMRFamide response remained unaltered.

The marked reduction in outward K currents during the presence of Cs^+ ions in the cell was used advantageously to voltage clamp the neurone at more positive potentials than was otherwise possible. The relationship between holding potential and response amplitude in the presence of intracellular Cs is relatively linear (Fig.3:18). The response remained an inward current over the entire range of holding potentials investigated (-70 to +20 mV). Extrapolation of a line fitted by linear regression to the data gave a theoretical reversal potential of +40 mV.

However, this value is unlikely to be a true indication of the reversal potential. The broken line in Fig.3:19. is the theoretical curve for a Na current displaying constant-field rectification (Goldman, 1943; Hodgkin and Katz, 1949). This curve, which fits the data better than the straight line, was obtained using an internal Na concentration of 3.8 mM (Meech and Thomas, 1977), an external Na concentration of 90 mM (c.f. Table 2:1), and a permeability constant for Na of 1.615×10^{-10} cm/s.

To summarize, the data presented above suggests that the depolarization elicited by FMRFamide is the result of an increase in conductance mainly to Na^+ ions. However, it is uncertain whether the channel activated by FMRFamide is highly selective for Na^+ ions or whether it is also permeable to other ions such as K^+ and Ca^{2+} ions. It is unlikely to have similar permeability ratios as the ACh activated cation selective channel present in these neurones. Interestingly, a high concentration (0.2 mM) of d-tubocurarine does not affect the response (Cottrell, 1983); a result which was confirmed in this study. This compound blocks depolarizing responses induced by many substances in molluscan neurones (Carpenter, Swann and Yarowsky, 1977).

Fig.3:18. Relationship between holding potential and the amplitude of the FMRFamide induced inward current in the presence of intracellular Cs^+ ions. The straight line was fitted by linear regression. Extrapolation of this line gives a reversal potential for the response of +40 mV.

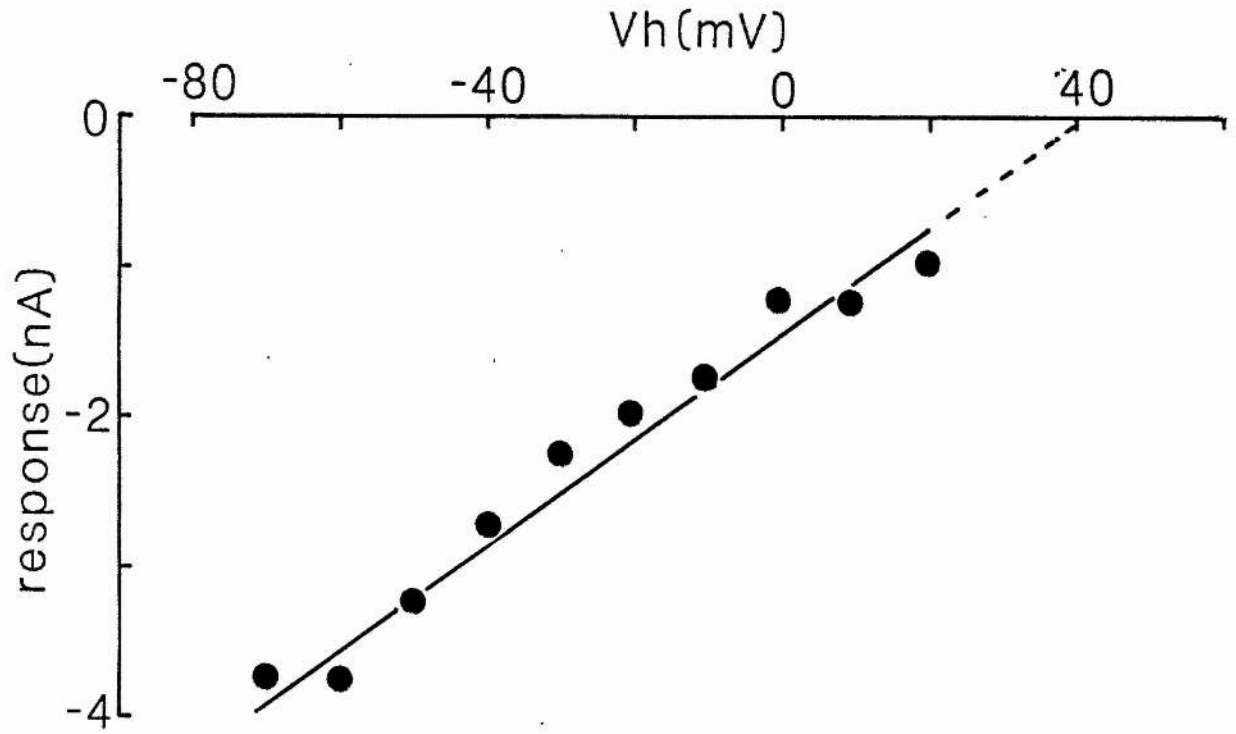
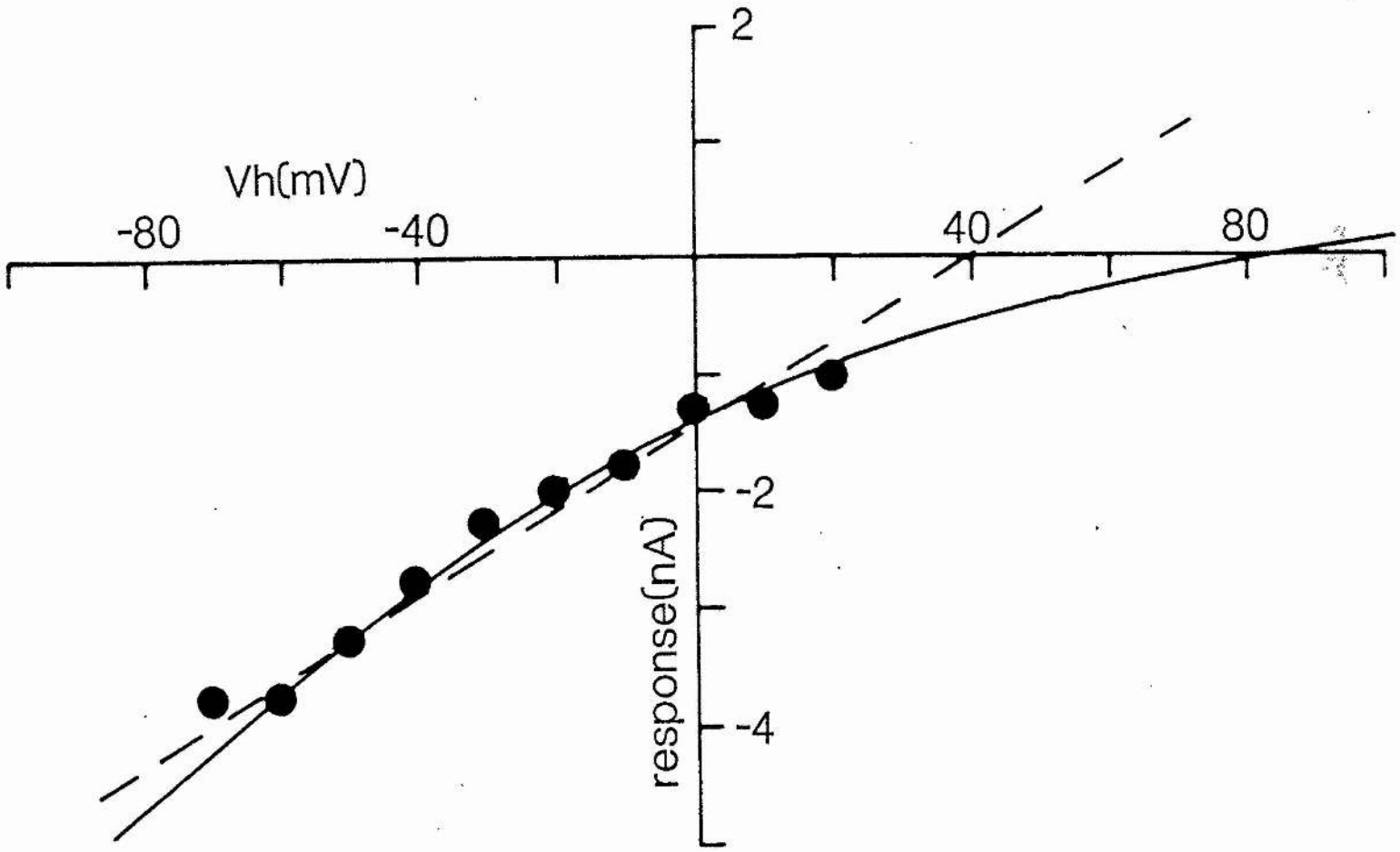

Vh(mV)
-80
-40
0
40
0
-2
-4
response(nA)

Fig.3:19. A fit of the GHK equation to the data of Fig.3:18., assuming that the response is a pure increase in gNa. The values used for the internal and external Na concentration were 3.8 mM and 90 mM respectively and a value of 1.615×10^{-10} cm/s was used for the permeability of the FMRFamide induced increase in gNa. This curve appears to fit the data better than a straight line. Extrapolation of this curve gives a reversal potential for the response of +80 mV, which is essentially equal to E_{Na}.


Vh(mV)
-80
-40
40
80
2
-2
-4
response(nA)

3.5 MULTIPLE RESPONSES ON SINGLE NEURONES.

3.5.1 A COMBINATION OF THE INCREASE IN gK AND THE INCREASE IN gNa CAN OCCUR ON SINGLE NEURONES.

The F2 neurone in the right parietal ganglion was depolarized by locally applied FMRFamide. Like the response described above, repeated application of FMRFamide led to a desensitization of the response (Fig.3:20). The reversal potential of the response to FMRFamide in the F2 neurone was, however, much more negative than that described above. Furthermore, it was variable from one F2 neurone to another, ranging from -20 to -55 mV. The relationship between response amplitude and holding potential was non-linear (Fig.3:21). During the action of FMRFamide on the F2 neurone, there was an increase in membrane conductance. This was indicated by an increase in current deflections resulting from command pulses applied during voltage clamp.

Exposure of the preparation to zero Na solution abolished the inward current component such that the reversal potential of the remaining response was similar to E_K (Fig.3:22). The relationship between membrane potential and response amplitude after removing Na was similar to that observed for the increase in gK elicited by FMRFamide. This result is consistent with the

Fig.3:20. Voltage recording of the response of an F2 neurone to ionophoretically applied FMRFamide (▲). Repeated application of FMRFamide led to a desensitization of the response. The response returned after a short period in the absence of peptide.

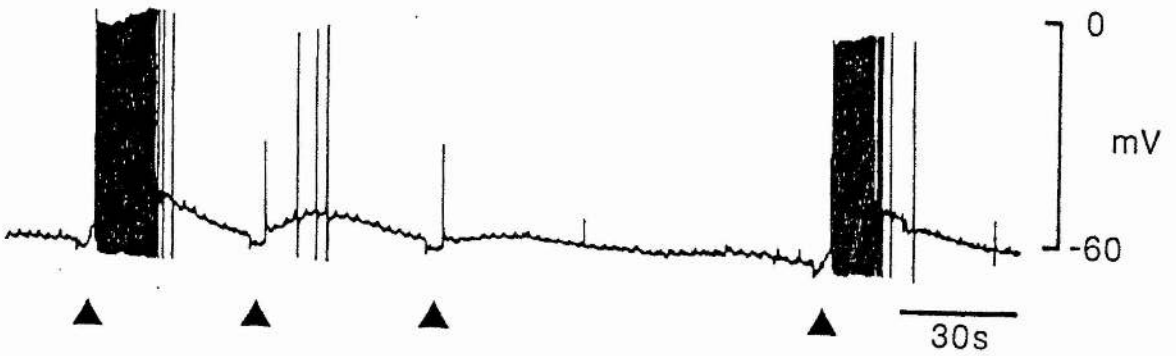
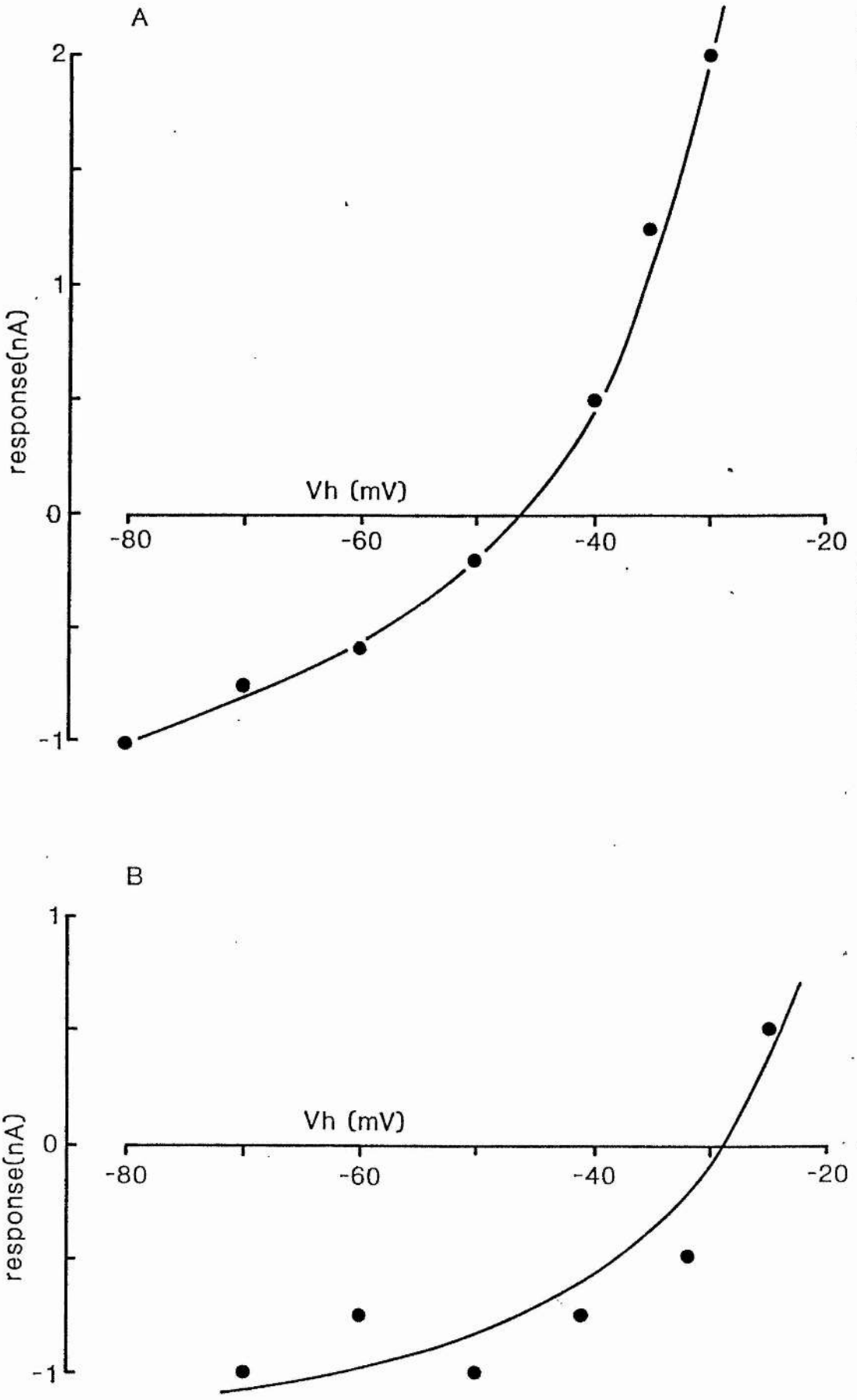

0
mV
-60
30s

Fig.3:21. The relationship between FMRFamide response amplitude and holding potential in two F2 neurones. In A, the reversal potential of the response was about -47 mV, while in B it was about -29 mV. Variation of the reversal potential of the responses of F2 neurones to FMRFamide was common.


A
2
1
0
-1
response(nA)
Vh (mV)
-80
-60
-40
-20
B
1
0
-1
response(nA)
Vh (mV)
-80
-60
-40
-20

FMRFamide response of the F2 neurone being a combination of an increase in gNa and an increase in gK.

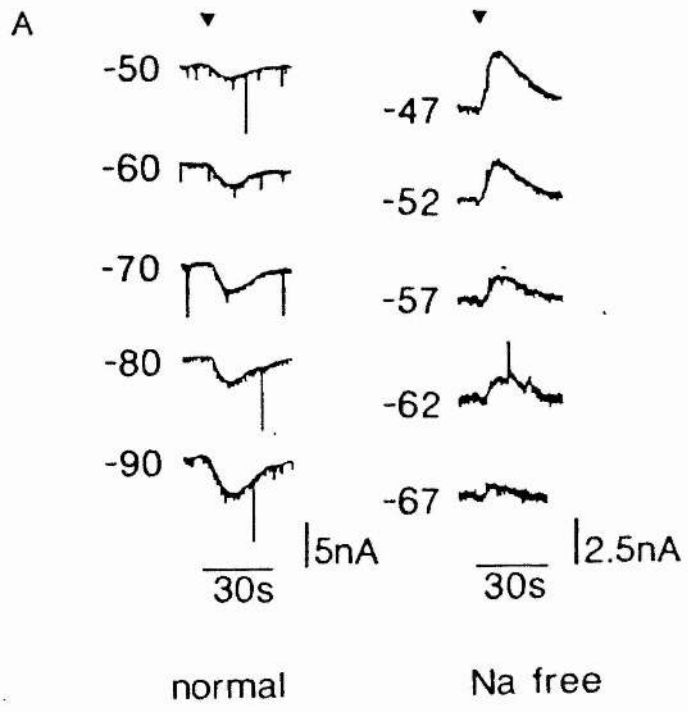
3.5.2 THE EFFECT OF K CHANNEL BLOCKERS ON THE RESPONSE OF THE F2 NEURONE TO FMRFamide.

The variability of the reversal potential observed between preparations implied that there was more than one type of channel associated with the conductance increase seen in the F2 neurone. This was further supported by the action of TEA on the FMRFamide induced response of the F2 neurone. Physiological solution containing 30 mM TEA increased the inward current observed and shifted the reversal potential to less negative values (Fig.3:23). This effect of TEA is explained by the presence of two populations of channels activated by FMRFamide on the F2 neurone; one type associated with the increase in gK by FMRFamide is sensitive to TEA, the other type associated with the increase in gNa is insensitive to TEA.

Additional evidence supporting a combination of the increase in gK and the increase in gNa occurring on the F2 neurone was the effect of intracellular Cs^+ ions on the overall response. Using a CsCl recording electrode as described above, the effect of Cs on the response was to abolish the outward K component while leaving the Na component intact. Under these conditions the inward current in the F2 neurone was identical to that reported

for the pure depolarizing component described above.

Fig.3:22. The effect of zero Na solution on the response of the F2 neurone to ionophoretically applied FMRFamide. A, current records obtained from an F2 neurone voltage clamped at various holding potentials in the presence and absence of Na ions. The holding potential is indicated in mV by each record. B, graphical presentation showing the difference between the amplitude of the current responses in normal (●) and in zero Na (■) solution at various holding potentials.


A
-50
-60
-70
-80
-90
-47
-52
-57
-62
-67
5nA
30s
2.5nA
30s
normal
Na free

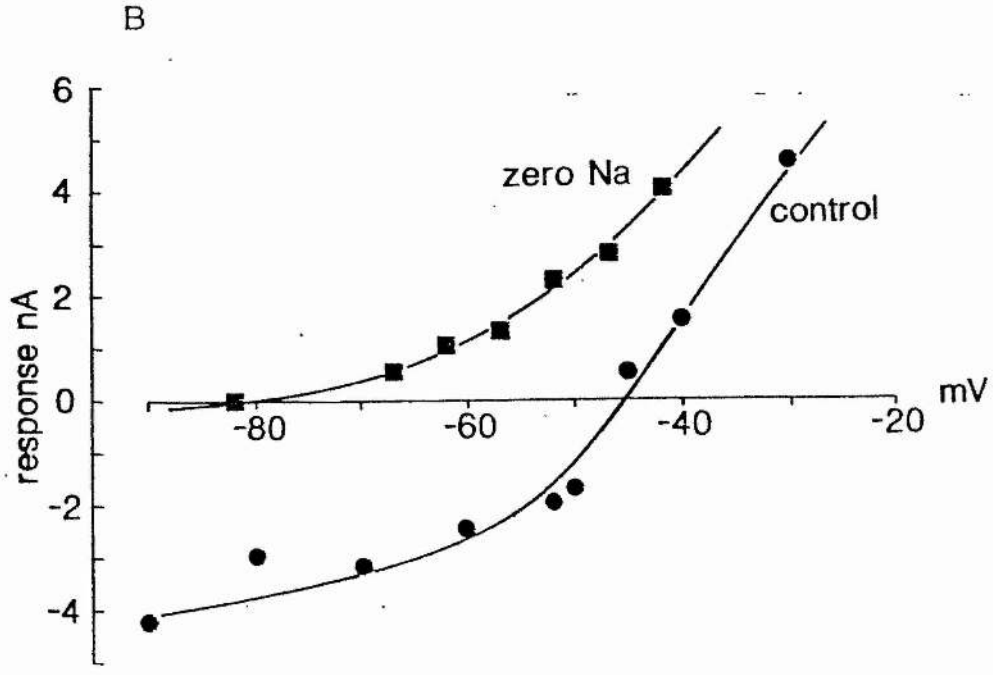
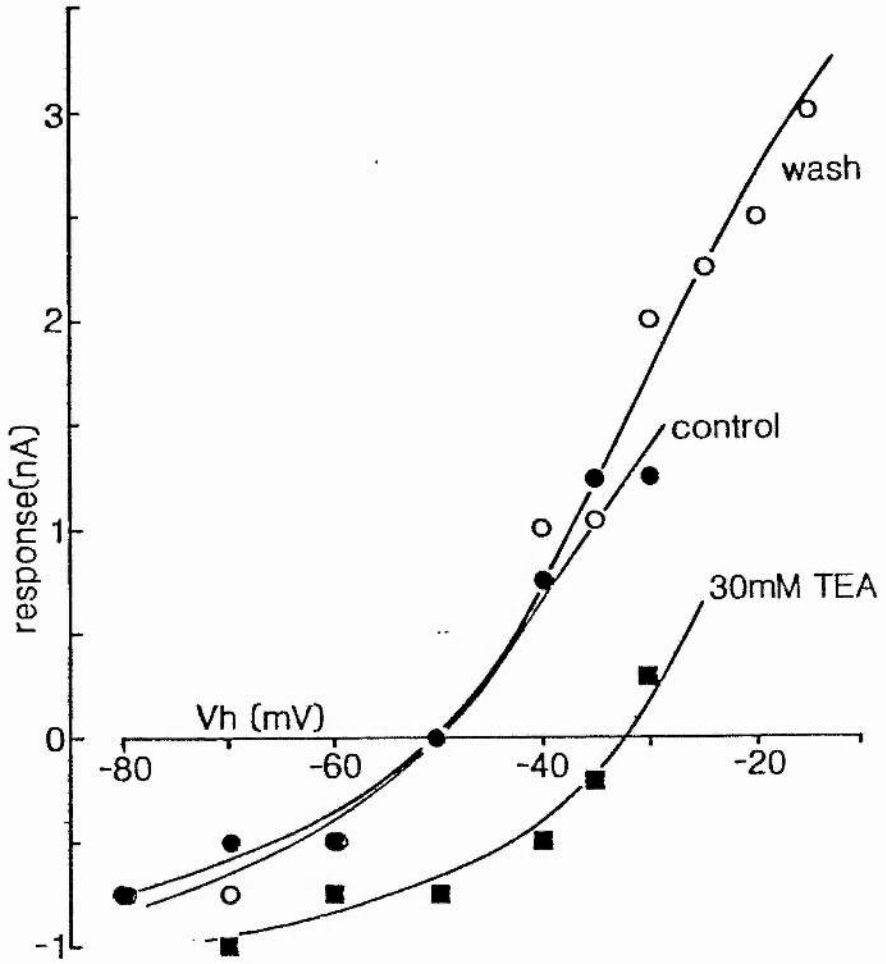

B
6
4
2
0
-2
-4
response nA
zero Na
control
-80
-60
-40
-20
mV

Fig.3:23. The effect of 30 mM TEA on the response of an F2 neurone to ionophoretically applied FMRFamide. TEA markedly reduced the outward K component of the FMRFamide induced current and shifted the reversal potential from -50 to -33 mV.


3
2
1
0
-1
response(nA)
Vh (mV)
-80
-60
-40
-20
wash
control
30mM TEA

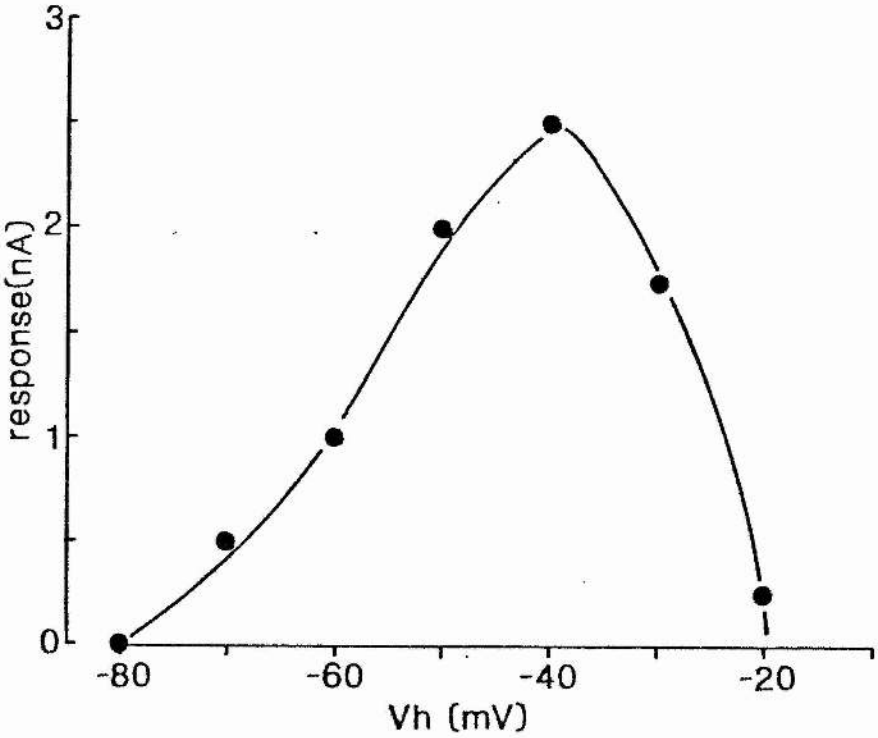
3.6 THE VOLTAGE DEPENDENT RESPONSE.

3.6.1 PROPERTIES OF THE VOLTAGE DEPENDENT RESPONSE.

Application of FMRFamide onto the C1 neurone produced, in addition to the increase in gK, a voltage dependent inward current at holding potentials more positive than -40 mV. Frequently, this response was only detected as a decrease in the outward FMRFamide induced K current as the C1 was depolarized (Fig.3:24). The simultaneous activation of an outward and inward current by FMRFamide at these potentials made the analysis of the mechanism of action very complicated. However, Cottrell (1982a) reported that an analogue of FMRFamide, YGGFMRFamide, was more potent at producing the inward current response. YGGFMRFamide was therefore used to study this response. 5-HT also produces a similar voltage dependent response in the C1 neurone (Cottrell, 1981).

In many preparations, careful positioning of the ionophoretic (or pressure ejection) pipette resulted in YGGFMRFamide eliciting the voltage dependent inward current only. The relationship between the amplitude of the response and holding potential is shown in Fig.3:25. The response could not be reversed, even at holding potentials of -60 to -70 mV. As membrane potential was made more positive than -40 mV, the response appeared, and

Fig.3:24. Ionophoretic application of FMRFamide onto the C1 neurone at potentials more positive than -40 mV frequently resulted in a decrease in the amplitude of the outward current response. Here the amplitude of the response in a C1 neurone is plotted against holding potentials between -80 and -20 mV.


3
2
1
0
response(nA)
-80
-60
-40
-20
Vh (mV)

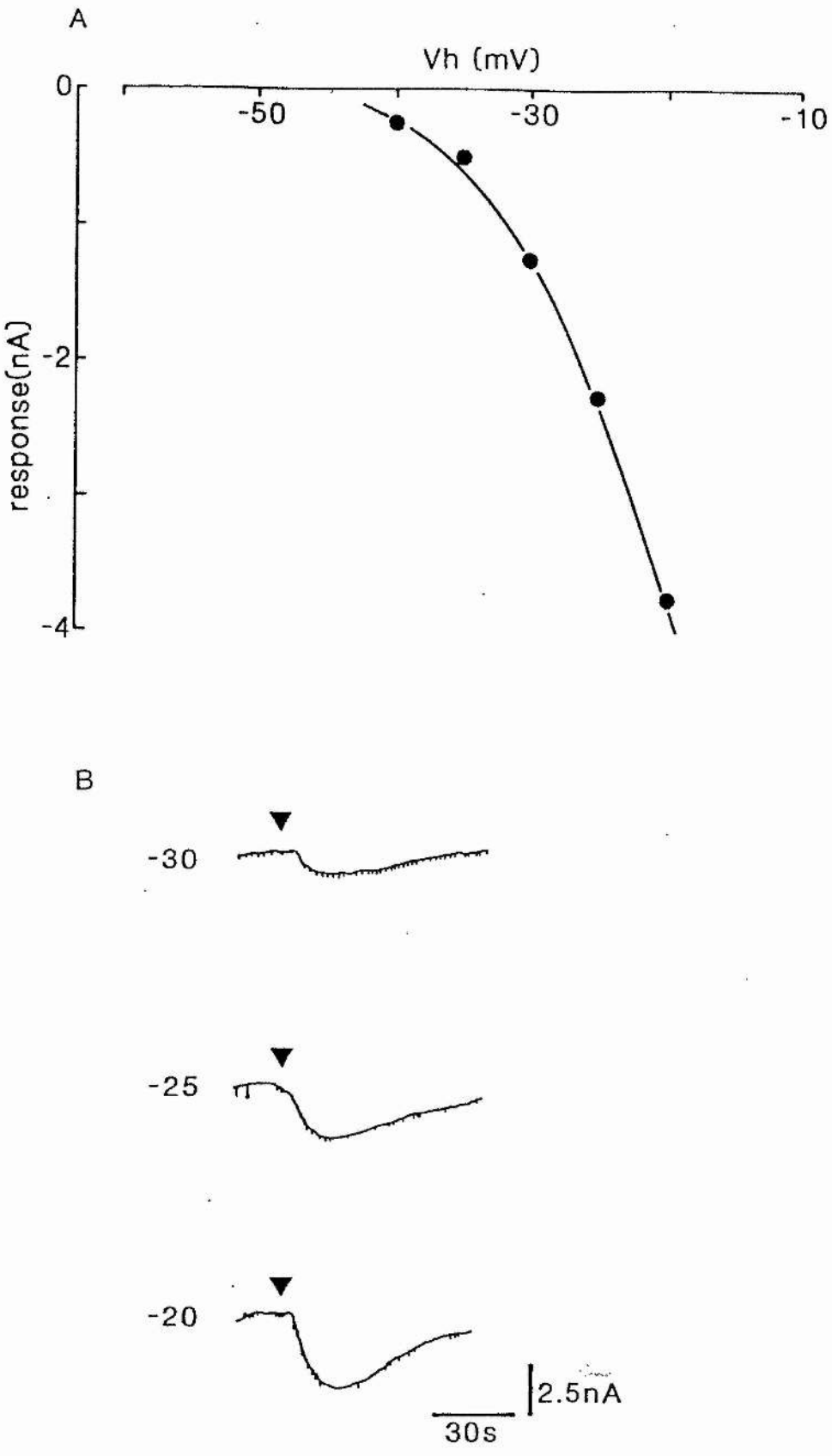
with further depolarization there was a marked increase in the amplitude of the inward current. These observations show why the term 'voltage dependent' is used to describe this response.

3.6.2 IONIC MECHANISM OF THE RESPONSE.

During the voltage dependent response, there was a decrease in the size of current deflections resulting from command pulses applied to the C1 neurone. As mentioned previously, care must be taken in the interpretation of such data, especially with regard to voltage dependent systems. There are two possible mechanisms which would explain this response: (1) a voltage dependent increase in conductance to Na^+ and/or Ca^{2+} ions, (2) a decrease in a voltage dependent outward current such as a Cl^- or K current. Both of these situations would lead to a reduction in the amplitude of the current deflections as described above.

It is unlikely that either Na^+ or Cl^- ions are involved in the response as replacing NaCl with sucrose or glucoseamine hydrochloride has been shown not to affect this response (Cottrell, 1982a). The inward current may result from an increase in conductance to Ca^{2+} ions, a decrease in conductance to K^+ ions or a combination of both.

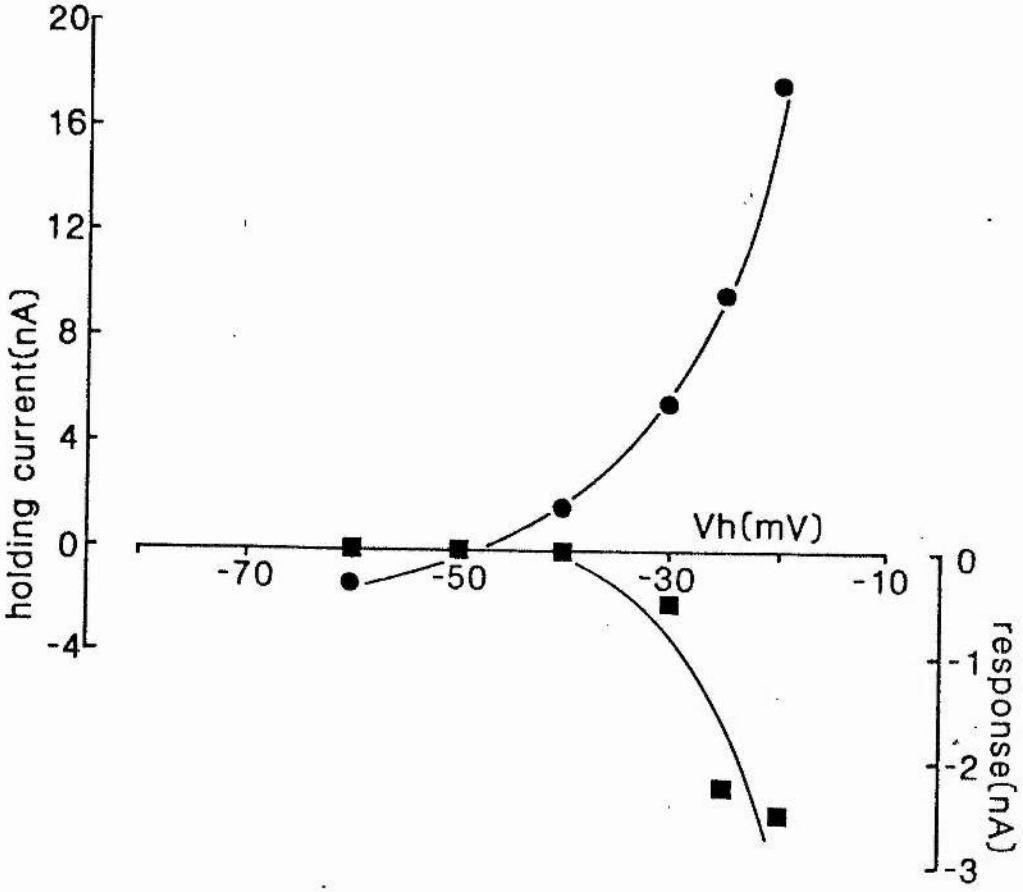
Fig.3:25. The voltage dependent response activated by YGGFMRFamide. A, relationship between response amplitude and holding potential. B, Examples of the inward current elicited at holding potentials of -30, -25 and -20 mV.


A
Vh (mV)
0
-50
-30
-10
response(nA)
-2
-4
B
-30
-25
-20
2.5nA
30s

Cottrell (1982a) has shown that this response is blocked by 1.5 mM Co^{2+} ions, and also when Ba was substituted for most of the Ca. Co^{2+} ions have been shown to block Ca currents in Helix neurones (Akaike, Lee and Brown, 1978; Barnes, Cottrell and Dunbar, 1985). However, it is unlikely that the response consists of an increase in gCa alone since Ba, which can permeate Ca channels quite adequately (Akaike, Lee and Brown, 1978), blocked the response. Ba^{2+} ions have been shown to block K currents in molluscan neurones (Gorman and Hermann, 1979), thus these observations by Cottrell are consistent with a YGGFMRFamide induced decrease in a Ca activated K current.

A comparison of the voltage sensitivity of the response with the steady state I-V curve of the C1 neurone is shown in Fig.3:26. The occurrence of the voltage dependent response is closely paralleled by the outward rectification present as the neurone is voltage clamped at depolarized potentials. It is therefore conceivable that the response may be the result of a decrease in one or more of the components of this rectification. A possible effect on the Ca activated K current was investigated further by increasing the intracellular Ca concentration.

Fig.3:26. A comparison of the amplitude of the voltage dependent response to YGGFMRFamide with the steady state I-V relationship of a C1 neurone. The steady state current was measured just before the application of YGGFMRFamide. ■, amplitude of the YGGFMRFamide response; ●, amplitude of the steady state current.


20
16
12
8
4
0
-4
holding current(nA)
Vh(mV)
-70
-50
-30
-10
0
-1
-2
-3
response(nA)

3.6.3 Ca INJECTION EXPERIMENTS.

The role of Ca in the generation of the response was investigated by injecting Ca^{2+} ions into the cell. The effect of peptide application was compared before and after Ca injection. The results presented here confirm and extend the observations made by Cottrell (1982a).

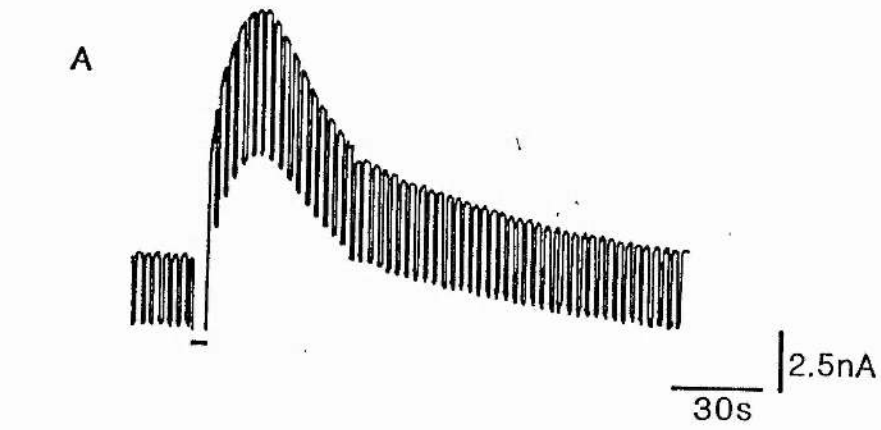
Ca was injected into the cell by ionophoresis. A second microelectrode, filled with 0.5 M $CaCl_2$, was inserted into the cell, and to inject Ca^{2+} ions a current of 40 to 60 nA for a duration of 5 to 15s was passed with respect to another electrode placed in the recording medium. The Ca injection was performed during voltage clamp, therefore limiting the change in membrane potential to a few millivolts. The injection of Ca^{2+} ions into the cell caused an increase in the outward current and an increase in membrane conductance (Fig.3:27). Similar results were reported by Meech (1974) and Meech and Standen (1975) who showed that the increase in membrane conductance was due to an increase in K permeability. The current elicited by Ca injection into the C1 neurone was reversed to an inward current when the neurone was clamped at potentials negative to E_K. This is consistent with K^+ ions carrying the Ca induced current in the C1 neurone.

To test whether YGGFMRFamide suppressed a Ca activated K current, the effect of ionophoretically applied YGGFMRFamide was compared before and after Ca injection. If the response was a suppression of a Ca activated K current, then the amplitude of the response would be expected to be limited by the amount of Ca activated K current present. The experiments were performed at a holding potential of -40 mV. At this potential the YGGFMRFamide response was very small and there is only minimal activation of the Ca dependent K current (Meech and Standen, 1975; Lux and Hofmeier, 1982).

The result of such an experiment is shown in Fig.3:28A. The YGGFMRFamide response before Ca injection was negligible, however, a substantial response was observed after Ca injection. The response amplitude appeared to increase with the amplitude of the Ca evoked current (Fig.3:28B). This result is consistent with a suppression of a Ca activated K current. However, out of 70 Ca injection experiments, only 8 resulted in an increased YGGFMRFamide response.

A major problem with the Ca injection experiments was the relatively rapid decrease of the Ca induced outward current. The injection of Ca in the presence of the metabolic inhibitor carbonyl cyanide m-chlorophenyl

Fig.3:27. The effect of Ca injection on the currents recorded from the C1 neurone under voltage clamp. A, a C1 neurone was held at -30 mV and regular hyperpolarizing command pulses to -35 mV were applied. The injection of Ca (60 nA during the period indicated by the bar) induced an outward current with a concomitant increase in the amplitude of the current deflections resulting from the command pulses. B, Ca injection in another C1 neurone at two different holding potentials. At -35 mV the current evoked by Ca injection was outward, at -90 mV the current was reversed to an inward direction. Ca injection: 60 nA during the period indicated by the bars.


A
2.5nA
30s

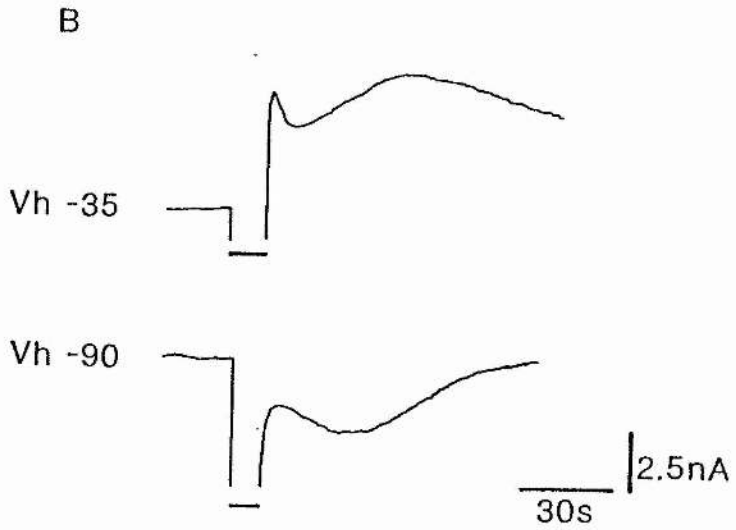
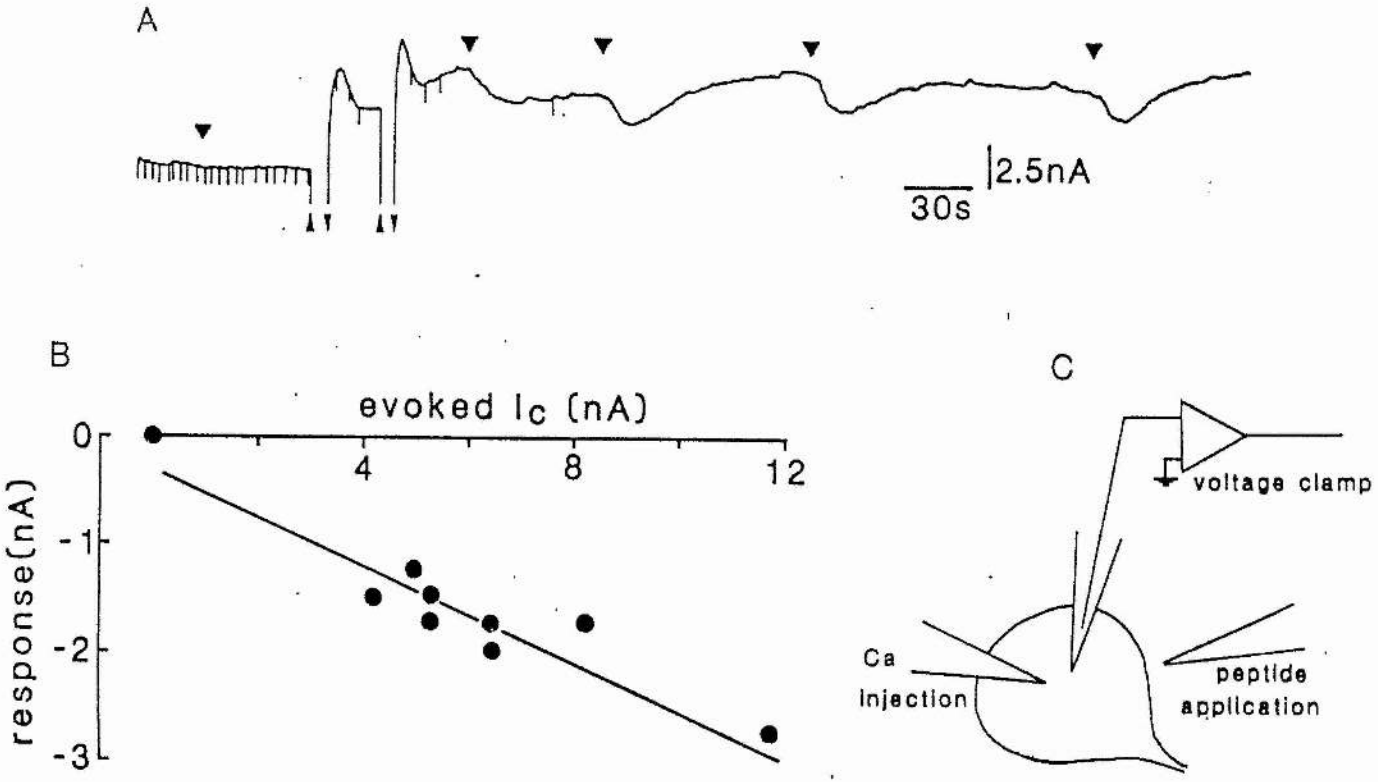

B
Vh -35
Vh -90
2.5nA
30s

Fig.3:28. The effect of ionophoresed YGGFMRFamide on the K current induced by injection of Ca^{2+} ions into the C1 neurone. A, a C1 neurone was voltage clamped at -40 mV and YGGFMRFamide was ionophoretically applied (▼). Ca was injected into the cell by passing 60 nA through the Ca electrode two times. The duration of the two injections are marked by the arrows and are evident in the current trace as rapid inward current deflections. Before Ca injection the response to YGGFMRFamide was negligible, however, after Ca injection there was a marked increase in both the outward current and the amplitude of the YGGFMRFamide response. Ionophoretic artefacts have been removed from this record. B, amplitude of the YGGFMRFamide induced response plotted against the outward current (Ic) evoked by Ca injection. The straight line is a fit to the data using regression analysis. C, a diagramatical representation of the experimental procedure.


A
2.5nA
30s
B
evoked Ic (nA)
0
-1
-2
-3
4
8
12
response(nA)
C
voltage clamp
Ca
injection
peptide
application

hydrazone (CCmP) has been shown to produce a more prolonged effect than that which is normally observed (Meech and Thomas, 1980). In an attempt to prolong the Ca evoked current in the C1 neurone, Ca was injected following exposure of the preparation to 10 uM CCmP. While this treatment was effective in prolonging the Ca evoked current, the response to YGGFMRFamide was completely abolished by CCmP (n=2). This suggested an involvement of a metabolic process in the generation of the voltage dependent response.

3.6.4 THE EFFECT OF Ca CHANNEL BLOCKERS.

Ca channel blockers have been shown to block the Ca activated K current in Helix (Meech and Standen, 1975) and Tritonia neurones (Thompson, 1977). If the response was the result of a decrease in a Ca activated K current, then compounds which block the Ca current would be expected to block the response. Both Co and verapamil have been shown to block the Ca current of Helix (Akaike, Lee and Brown, 1978; Barnes, Cottrell and Dunbar, 1985). However, only Co blocked the response (n=3, Fig.3:29), while 10 um verapamil left the response unaltered (n=2). The effect 1 mM Co had on the I-V curve was to reduce steady state outward currents appreciably (Fig.3:30). However, 10 um verapamil, in contrast to 1 mM Co, had no effect on the I-V curve of the C1 neurone (n=2, Fig.3:30). The difference observed between the effects on the I-V curve

of Co and verapamil suggests that Co may have an effect which is additional to the block of Ca channels. The observations described above imply that the voltage dependent response is not a suppression of a K current which is activated only by an influx of Ca across the cell membrane.

This voltage dependent response was more complicated than the increased conductance responses to FMRFamide. The response is a decrease in a K conductance and was only detected at potentials more positive than -40 mV. It was blocked by 1 mM Co but unaffected by the Ca channel blocker verapamil. Therefore the role of Ca in this response is unclear.

3.6.5 A STUDY OF THE RESPONSE AT THE SINGLE CHANNEL LEVEL.

The voltage dependent response in the C1 neurone has been studied using patch clamp methods (Cottrell, Davies and Green, 1984). Using a cell attached patch, the activity of single K channels was decreased when the FMRFamide analogue FnLRFamide was applied to the surface of the C1 neurone outwith the patch. These channels have been shown not to be Ca activated (Barnes, Cottrell and Dunbar, 1985). The method of application of the peptide, i.e. outwith the recorded patch of membrane, suggests that the effect of the peptide is mediated through a second messenger system. In Aplysia sensory neurones,

cAMP has been shown to reduce K channel activity (Siegelbaum, Camardo and Kandel, 1982) and in Helix an increase in cAMP reduces outward K currents (Deterre, Paupardin-Tritsch, Bockaert and Gerschenfeld, 1981). It is thus possible that a cyclic nucleotide may mediate the effects of YGGFMRFamide in producing the voltage dependent response in the C1 neurone.

3.6.6 THE EFFECT OF INCREASING CYCLIC NUCLEOTIDE LEVELS ON THE C1 NEURONE.

Exposure of the preparation to 1 mM theophylline reduced the the outward current of the C1 neurone. This was evident as a 'flattening' of the I-V curve (n=2, Fig.3:31). During the action of theophylline, the voltage dependent response was abolished. Theophylline inhibits cyclic nucleotide breakdown by interfering with phosphodiesterase enzymes. This result implies that there is a basal production of cyclic nucleotides in the C1 neurone, and that the outward current observed under voltage clamp conditions is sensitive to cyclic nucleotides.

Fig.3:29. The effect of 1 mM Co on the voltage dependent response. Application of YGGFMRFamide (▼) onto a voltage clamped C1 neurone produced the voltage dependent inward current, which was blocked after the preparation was exposed to 1 mM Co for 6 minutes. Full recovery was not obtained. The downward deflections in the traces are probably unclamped axon spikes. The holding potential in mV for each pair of recordings is indicated by the side.

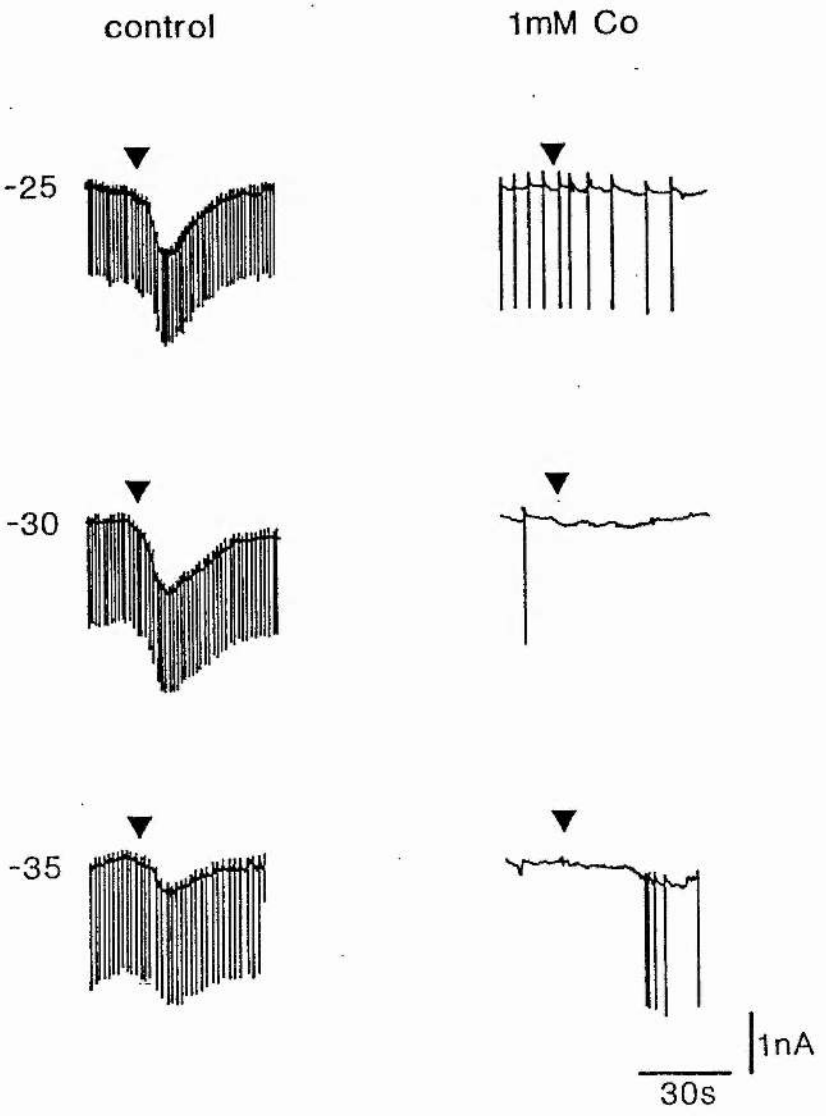

control
1mM Co
-25
-30
-35
1nA
30s

Fig.3:30. A, the effect of 10 uM verapamil on the steady state I-V curve of a C1 neurone. Over the range of potentials investigated, this concentration of verapamil had no effect on the background current (●, control; □, verapamil). B, 1 mM Co reduced the steady state outward current of a C1 neurone at potentials more positive than -40 mV (●, control; □, Co).

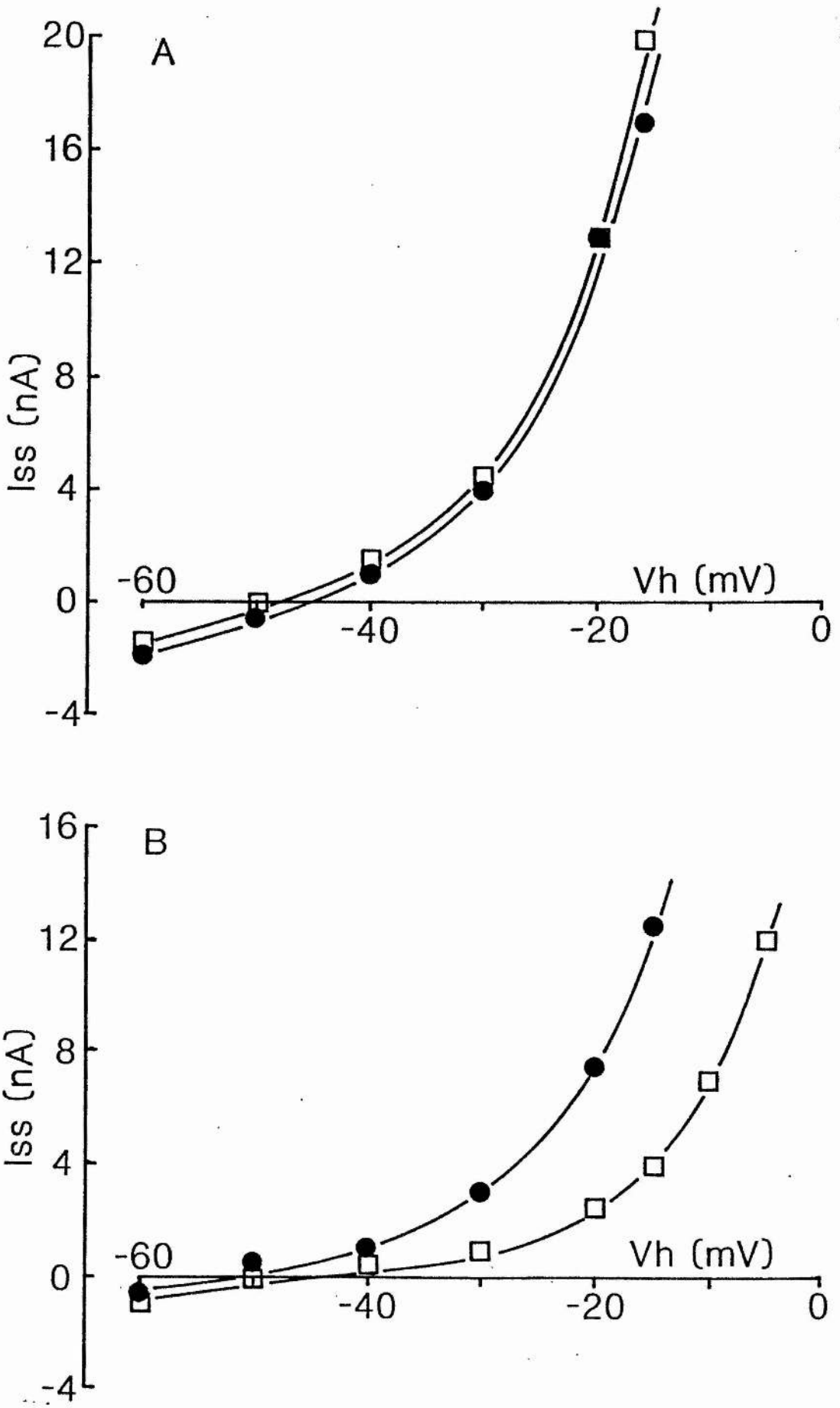
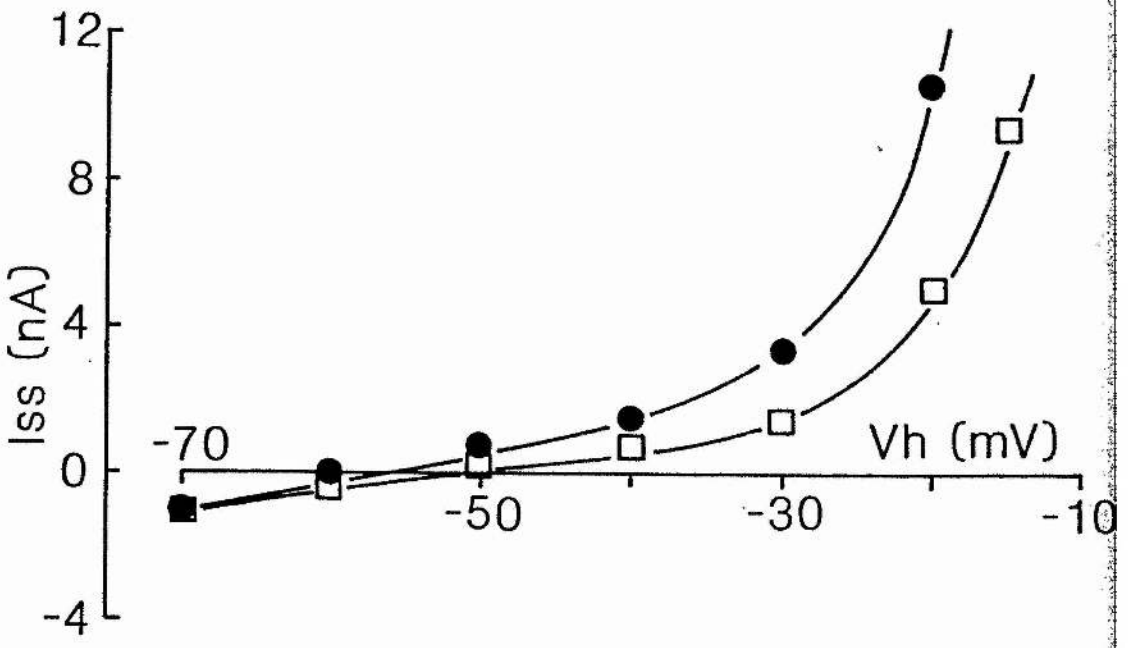

A
20
16
12
8
4
0
-4
Iss (nA)
-60
-40
-20
0
Vh (mV)
B
16
12
8
4
0
-4
Iss (nA)
-60
-40
-20
0
Vh (mV)

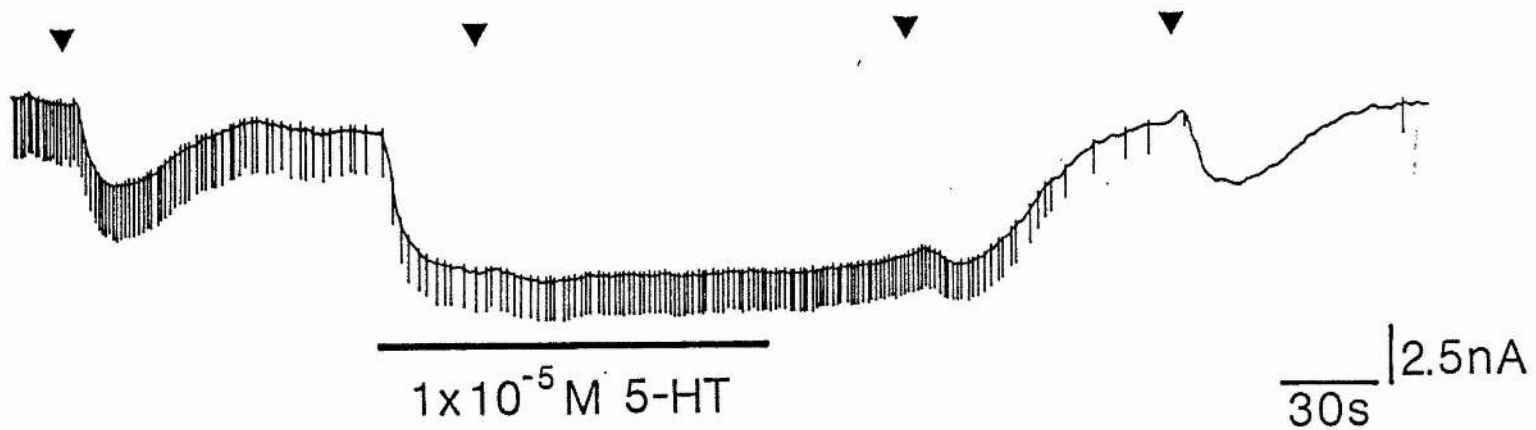
Fig.3:31. The effect of theophylline on the I-V curve of a C1 neurone. Exposure of the preparation to 1 mM theophylline reduced the steady state outward current observed at potentials more positive than -40 mV. ●, control; □, 1 mM theophylline.


12
8
4
0
-4
Iss (nA)
-70
-50
-30
-10
Vh (mV)

3.6.7 INTERACTION BETWEEN THE VOLTAGE DEPENDENT RESPONSE INDUCED BY 5-HT AND FMRFamide PEPTIDES.

The response of the C1 neurone to 5-HT has very similar properties to the peptide induced decrease in gK (Cottrell, 1981; Barnes, Cottrell and Dunbar, 1985). During the response to 5-HT in this neurone, the peptide induced response was attenuated or even absent (Fig.3:32). This suggested that the voltage dependent response to both 5-HT and FMRFamide peptides result from the reduction of the same K current, or that there is at least one common step in the production of the responses.

Fig.3:32. Interaction between the voltage dependent response to 5-HT and the voltage dependent response to the peptides in a C1 neurone voltage clamped at -25 mV. Application of pQDPFLRFamide (▼) induced the decrease in gK response (see Fig.3:47.). Perfusion of the recording chamber with $1x10^{-5}$ M 5-HT produced a marked inward current, during which application of pQDPFLRFamide failed to induce a response. The response to the peptide returned following recovery from the effect of 5-HT.


1x10-5 M 5-HT
30s
2.5nA

3.7 COMPARISON OF THE ACTIONS OF FMRFamide WITH THOSE OF FMRFamide ANALOGUES.

Three actions of FMRFamide on ionic currents have been described above, these are an increase in gNa, an increase in gK and a decrease in gK. Furthermore, these effects can be found reliably on at least three identified neurones:

C1 - increase in gK and decrease in gK

F2 - increase in gK and increase in gNa

E13 - increase in gNa only

These three identified neurones, together with a few unidentified neurones, provided a very useful system for comparing the actions of FMRFamide with other 'FMRFamide-like' peptides. A list of the FMRFamide peptides used in this comparative study is given below:

FMRFamide

YGGFMRFamide

FIRFamide

FLRFamide

PFLRFamide

GDPFLRFamide

pQDPFLRFamide

They were termed 'FMRFamide-like' because of the sequence Met(or Leu)-Arg-Phe-NH_2, which is important for biological activity (Price and Greenberg, 1980; Painter, Morley and Price, 1982).

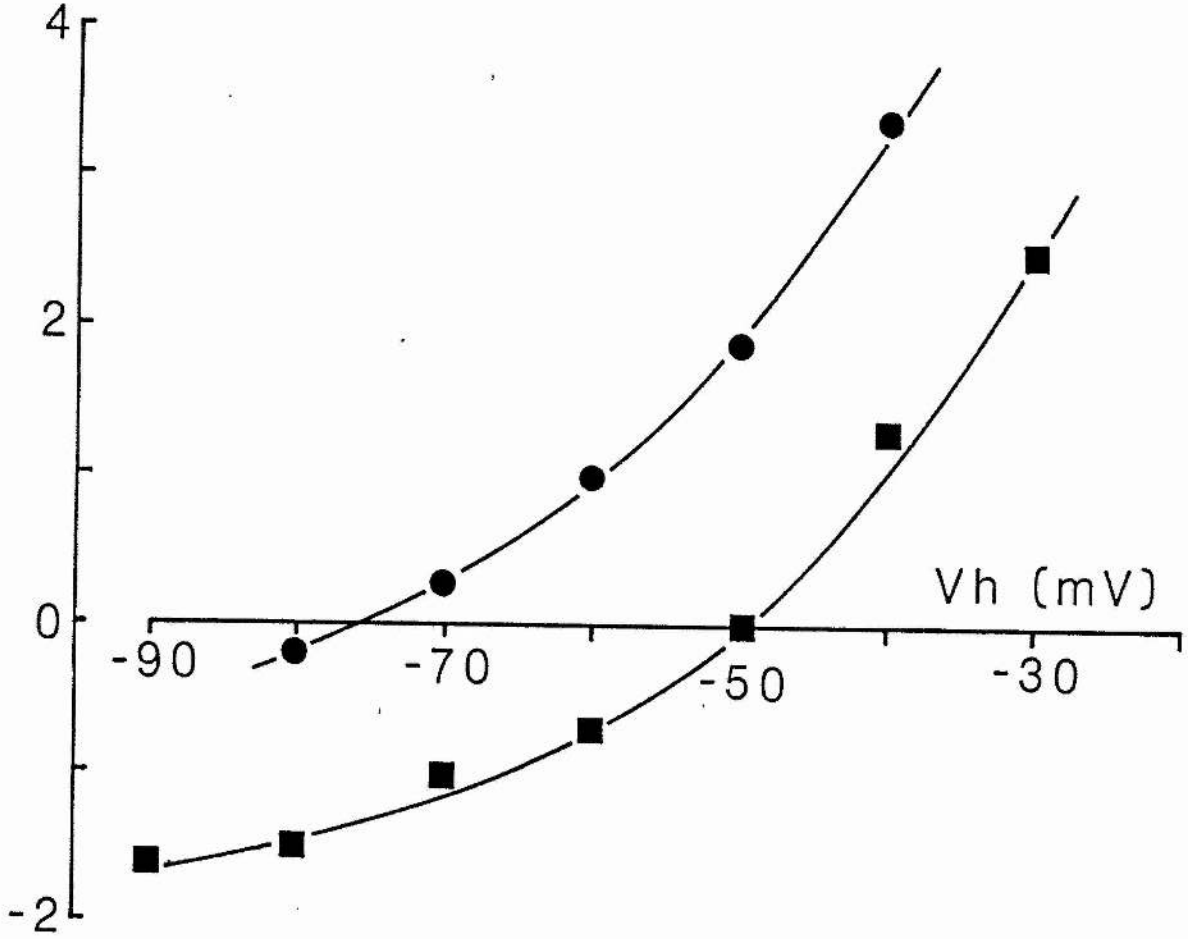
Two tetrapeptides, three heptapeptides and one pentapeptide were used in addition to FMRFamide in this investigation. Observations showed that both tetrapeptides had biological activity similar to FMRFamide, while the heptapeptides and pentapeptide had similar actions to each other which were different to those seen with the tetrapeptides (Cottrell and Davies, 1985). The results obtained imply that there are multiple receptor sites for FMRFamide peptides on Helix neurones.

3.8 SIMILARITIES BETWEEN FMRFamide AND TETRAPEPTIDE ANALOGUES.

3.8.1 FLRFamide AND FIRFamide INDUCE FMRFamide-LIKE RESPONSES.

As mentioned above, both FLRFamide and FIRFamide produced responses which were very similar to those induced by FMRFamide. Ionophoretic application of both FLRFamide and FIRFamide onto the C1 neurone induced a hyperpolarization. Many other unidentified cells in the suboesophageal ganglia responded to these two tetrapeptides with a hyperpolarization, which was observed as an outward current under voltage clamp conditions. This current was reversed to an inward current at membrane potentials more negative than -80 mV, which is close to E_K. Increasing the extracellular K^+ concentration from 5 to 15 mM changed the reversal potential of the response of a C1 neurone to FLRFamide from -76 to -50 mV (n=1, Fig.3:33). This 26 mV shift is very close to the theoretical value of 28 mV predicted by the Nernst equation for a pure K response. Since FMRFamide also produced an increase in gK in the C1 neurone, this result with FLRFamide was expected as it is conceivable that FLRFamide activates the same receptor as FMRFamide. FIRFamide was rather less potent at inducing the increase in gK.

Fig.3:33. The influence of extracellular K concentration on the increase in gK induced by ionophoretically applied FLRFamide onto a C1 neurone. The reversal potential of the response in normal medium (●, 5 mM K) was -76 mV. Increasing the external K concentration to 15 mM (■) changed the reversal potential to -50 mV.


4
2
0
-2
-90
-70
-50
-30
Vh (mV)

Application of both FLRFamide and FIRFamide onto the F2 and E13 neurones induced the increase in gNa. Like the FMRFamide induced increase in gNa, the response to FLRFamide and FIRFamide was rapidly desensitized by repeated application of the peptides. Exposure of the preparation to low Na solution reduced the amplitude of the response. A comparison of the increase in gNa induced by FLRFamide, FIRFamide and FMRFamide is shown in Fig.3:34.

The voltage dependent response present in the C1 neurone was very difficult to observe because of the masking of this response by the outward K current induced by these peptides. This problem of detecting the voltage dependent response was also present with FMRFamide (see page 76). A decrease in the activity of K channels has, however, been observed in a cell attached patch when FnLRFamide was applied to the cell surface outside the patch (Cottrell, Davies, and Green, 1984).

3.8.2 FMRFamide, FLRFamide AND FIRFamide ACTIVATE THE SAME RECEPTORS.

Many results imply that these three tetrapeptides act on the same receptors, thereby producing the same ionic responses. For example, all three peptides elicited an increase in gNa in the E13 neurone and an increase in gK

in the C1 neurone. A combination of an increase in gNa and an increase in gK was observed when FLRFamide was applied to the F2 neurone (n=2). The responses of a C1 neurone and an F2 neurone were unaltered following exposure of the preparation to a high Mg/low Ca solution (3.5 mM Ca, 15 mM Mg). This solution markedly reduced synaptic activity, suggesting that the responses observed were not due to an indirect action on the neurones. The response of the C1 neurone to FLRFamide resembled the time course and voltage dependency of the FMRFamide response (Fig.3:35). Furthermore, application of known concentrations of both peptides to the bath produced responses of a similar amplitude (n=1, Fig.3:36).

Recently Boyd and Walker (1985) have observed similar results when comparing FMRFamide and FLRFamide, and suggest that FLRFamide binds to the FMRFamide receptor. Also both FMRFamide and FnLRFamide are equipotent at inhibiting Lampsilis heart and contracting the Busycon radula protractor muscle, although there are differences in potency between the two peptides when tested on Macrocallista heart and Geukensia anterior byssus retractor muscle (Painter, Morley and Price, 1982).

Properties of the depolarization induced by FMRFamide are shared by FLRFamide and FIRFamide. Depolarizing (or inward current) responses induced by FLRFamide and FIRFamide only occurred on neurones which gave

Fig.3:34. Comparison of the effect of FMRFamide, FIRFamide and FLRFamide at inducing the increase in gNa. The responses were recorded from a neurone in the visceral ganglion, the recordings of the effect of FMRFamide and FIRFamide were from the same neurone; the FLRFamide response was recorded from the homologous neurone of another preparation. The holding potentials in mV are indicated by the side.

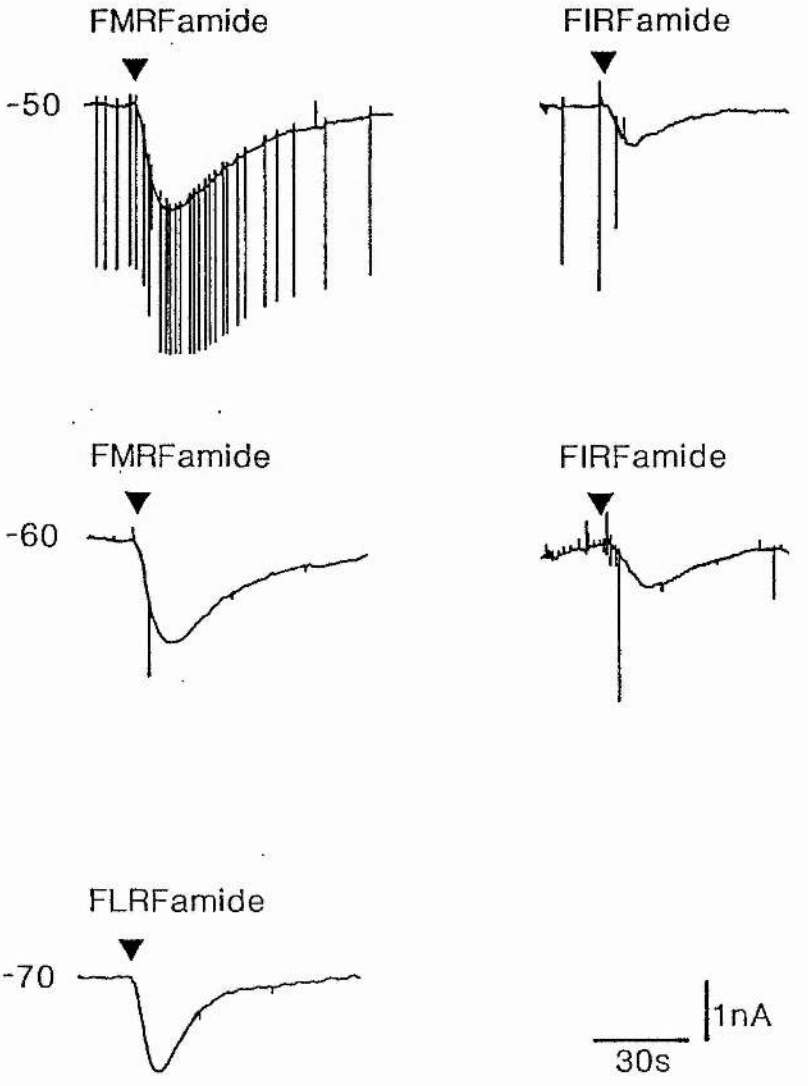

FMRFamide
FIRFamide
-50
FMRFamide
FIRFamide
-60
FLRFamide
-70
1nA
30s

Fig.3:35. Comparison of the responses induced by ionophoretic application of FMRFamide (●) and FLRFamide (□) onto a C1 neurone at various holding potentials. The voltage dependency and the amplitude and duration of the responses to the two peptides were similar.

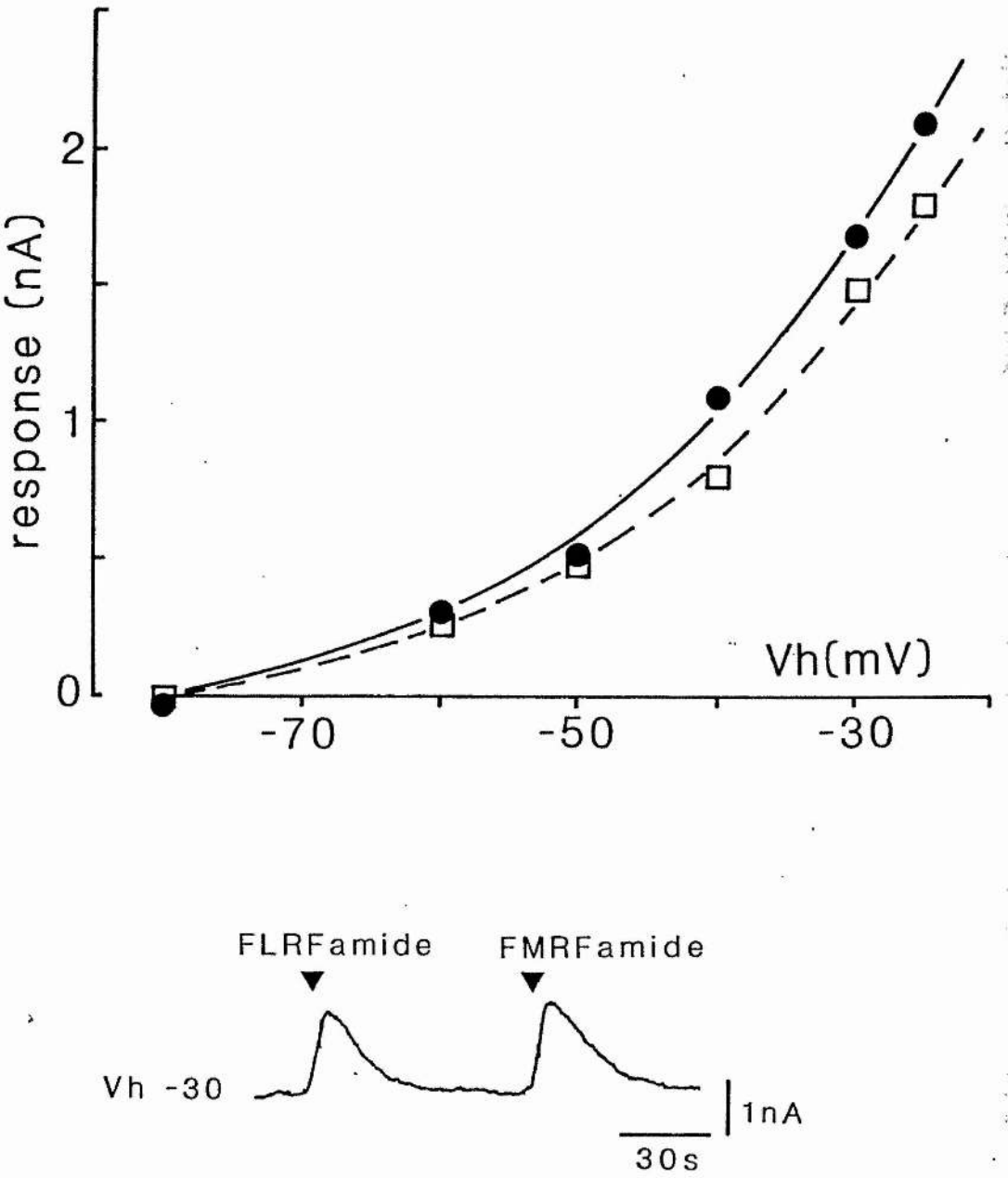
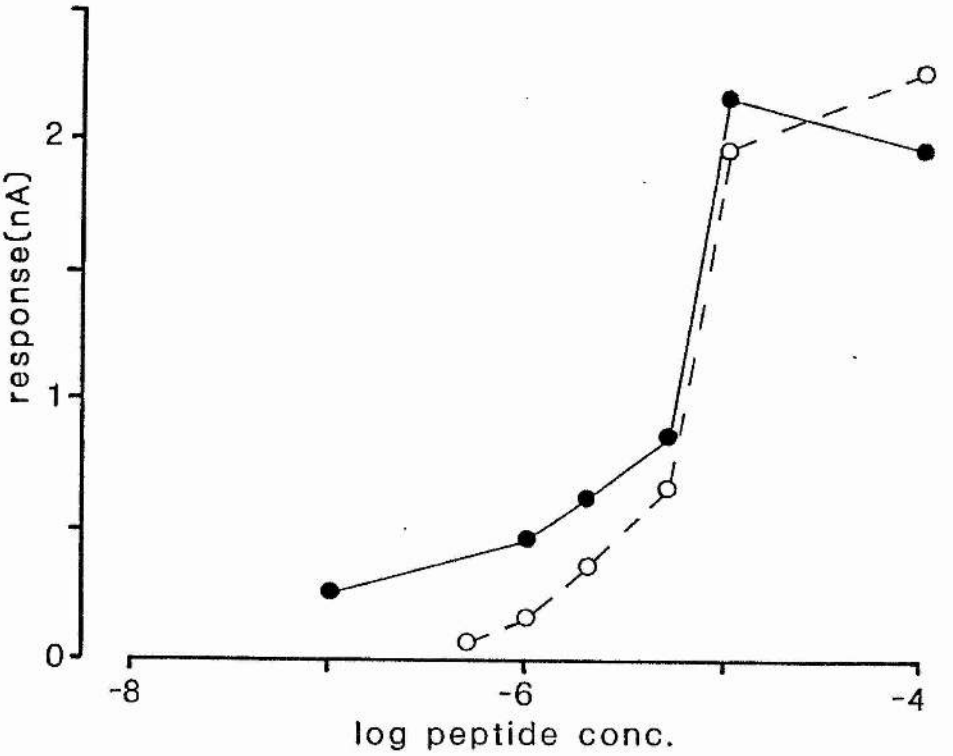

response (nA)
2
1
0
Vh(mV)
-70
-50
-30
FLRFamide
FMRFamide
Vh -30
1nA
30s

Fig.3:36. A plot of the amplitude of the K current generated in a C1 neurone by bath application of various concentrations of both FMRFamide (●) and FLRFamide (○). Both peptides exhibited a similar concentration dependency and maximum response. The C1 neurone was voltage clamped at -45 mV.


response(nA)
2
1
0
-8
-6
-4
log peptide conc.

depolarizing responses to FMRFamide. One of the major properties of this response was the rapid desensitization which occurred with repeated application of peptide (Fig.3:37). If these tetrapeptides were all acting on the same receptor to produce this response, then cross-desensitization would be expected to occur between them. This was investigated by ionophoresis of two peptides onto the same neurone. Such an experiment conducted with FMRFamide and FIRFamide is shown in Fig.3:38. Both peptides induced an inward Na current in a visceral ganglion neurone voltage clamped at -60 mV. The response was desensitized by three successive ionophoretic pulses of FMRFamide, ionophoretic application of FIRFamide following the FMRFamide induced desensitization failed to produce a response. The responses to FMRFamide and FIRFamide returned after a short period in the absence of peptide. In the same neurone, the FMRFamide response was virtually abolished following a FIRFamide induced desensitization. Similar cross-desensitization was also observed between FLRFamide and FMRFamide (n=2).

This rapid cross-desensitization observed between these three peptides, together with the similarity of their respective responses on identified neurones, substantiates the view that they activate the same receptors.

Fig.3:37. Similarity between the desensitization of the increase in gNa induced by FMRFamide and FLRFamide. A, a neurone in the visceral ganglion responding to FMRFamide was voltage clamped at a holding potential of -50 mV. Application of FMRFamide in rapid succession resulted in desensitization which began to recover after a short period in the absence of peptide (c.f. Fig.3:15). B, the homologous neurone in a different preparation voltage clamped at -70 mV responded in a similar way to applications of FLRFamide.

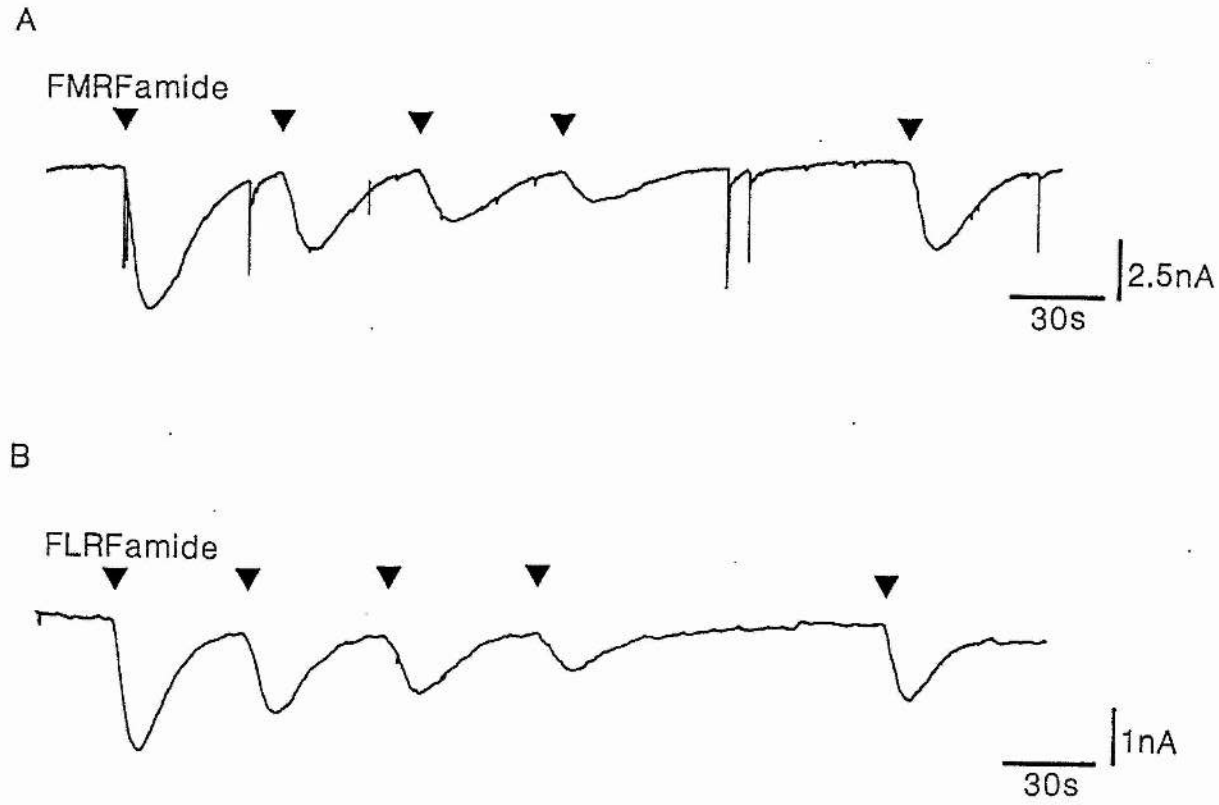
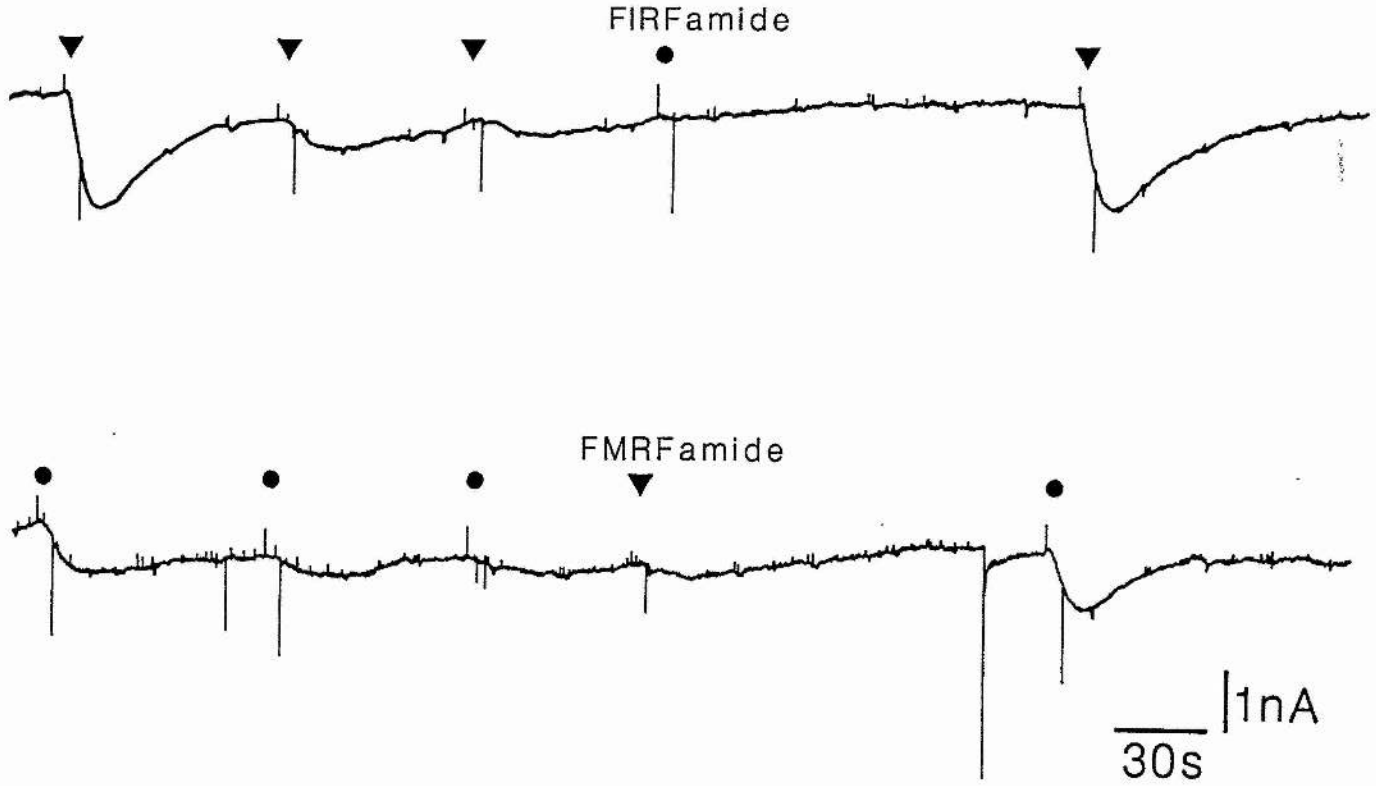

A
FMRFamide
2.5nA
30s
B
FLRFamide
1nA
30s

Fig.3:38. Cross-desensitization of the increase in gNa by FMRFamide and FIRFamide. Both traces were obtained from the same neurone in the visceral ganglion. The neurone was voltage clamped at a holding potential of -60 mV. The top record shows that desensitization of the response by ionophoretic application of FMRFamide (▼) resulted in a desensitization of the cell to FIRFamide (●). The bottom record, obtained a few minutes afterwards, shows the converse situation where FIRFamide application led to a desensitization to FMRFamide.


FIRFamide
FMRFamide
1nA
30s

3.9 ARE THE RECEPTORS MEDIATING THE INCREASE IN gNa AND THE INCREASE IN gK DIFFERENT?

The response of the F2 neurone to FMRFamide involved an increase in both gNa and gK. The variability of the reversal potential, and the results obtained with K channel blockers was interpreted as a simultaneous activation of the increase in gNa and the increase in gK responses by FMRFamide. This raised the interesting question whether the receptors associated with the two responses are different. The possibility that the receptors are different was investigated by obtaining dose response relationships for the FMRFamide induced increase in gK and the FMRFamide induced increase in gNa.

FMRFamide was applied by injecting quantities of between 5 and 20 µl of known concentration to the bath using a Gilson pipette. Initially the volume of the bath was 0.8 ml, this size of bath resulted in inadequate mixing of the injected peptide solution, which was tested by injecting volumes of ink. When the volume of the bath was reduced to 0.1 ml, mixing of the injected solution was much more satisfactory.

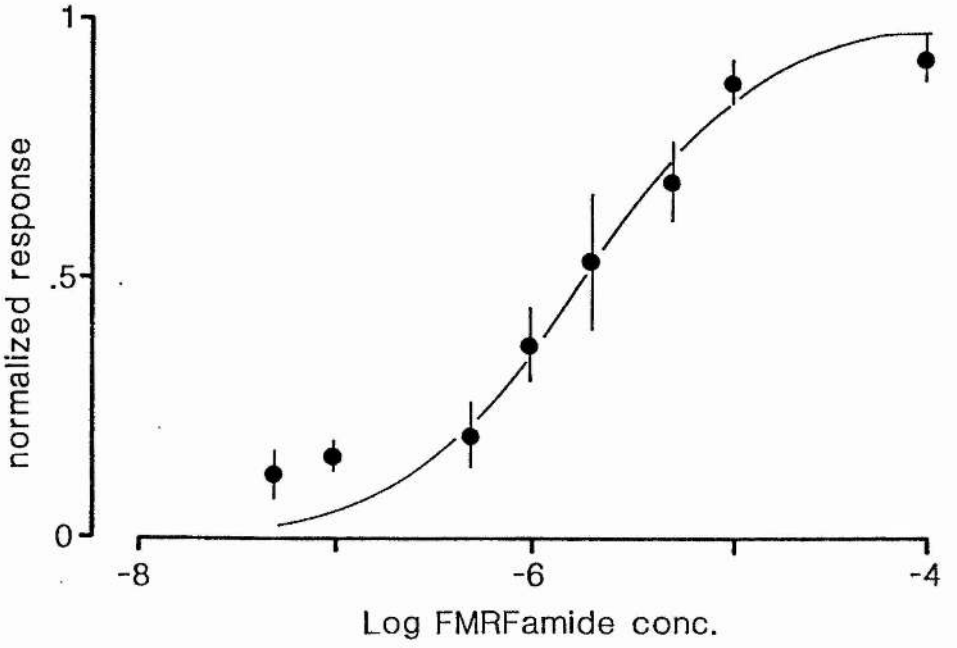
At membrane potentials more negative than -40 mV, the C1 neurone responded to FMRFamide with a pure increase in gK. The C1 neurone was therefore used to obtain the dose response relationship for the FMRFamide activated increase in gK. During application of FMRFamide, the flow of solution through the bath was stopped. After application of FMRFamide, the induced response reached a steady state, the value of which was taken as the response amplitude for that particular concentration of FMRFamide. Following the response, the preparation was washed by commencing the flow of solution. Using this method the threshold for the activation of the response was between $5x10^{-8}$ and $1x10^{-7}$ M, with maximal activation occurring around $1x10^{-5}$ M (Fig.3:39). Since the increase in gK was not desensitized, a maximal response could be obtained.

The F2 neurone was the most readily identifiable neurone displaying the increase in gNa response. Therefore this neurone was used to obtain the dose response relationship for the activation of the increase in gNa by FMRFamide. To eliminate any contamination by the increase in gK which also occurred in the F2 neurone, the neurone was voltage clamped at -70 mV, which is close to E_K. Because the FMRFamide induced increase in gNa is rapidly desensitized, a full dose response relationship could not be obtained. However, a limited range of responses at various concentrations was obtained

Fig.3:39. Dose response relationship for the FMRFamide activation of the increase in gK. The data from five experiments have been pooled, the bars represent the standard errors. The solid line is computed from:

$$N = C/(C + Kd)$$

where N is the normalized response, C is the concentration of peptide and Kd is the dissociation constant of the receptor for which a value of 1.8×10^{-6} M was used to generate this curve.

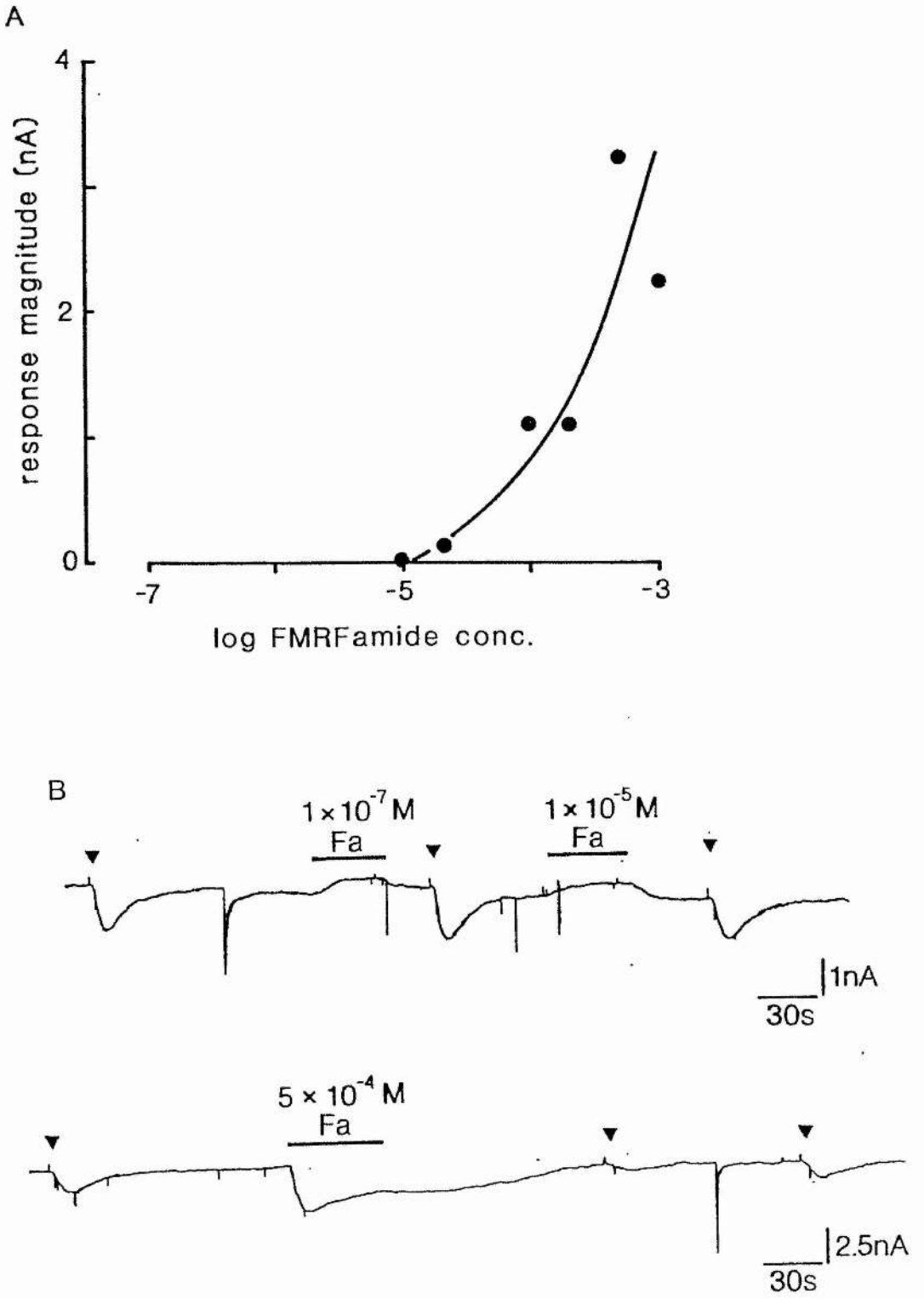

1
.5
0
normalized response
-8
-6
-4
Log FMRFamide conc.

(Fig.3:40A). Note that the threshold concentration for the activation of this response, at $1x10^{-5}$ M, was about one hundred times higher than the threshold concentration for the activation of the increase in gK (n=1).

It could be argued that the threshold value obtained for this response was artificially high as a result of desensitization. To test this hypothesis, a control was performed whereby FMRFamide was ionophoresed onto the cell in addition to being applied to the bath. This method allowed a check to be made on the desensitization state of the response following application of the peptide to the bath. The ionophoretic response to FMRFamide was not desensitized following applications of low concentrations of FMRFamide to the bath, and appreciable desensitization did not occur until a concentration greater than $1x10^{-5}$ M was applied (Fig.3:40B). Therefore the high threshold value for the activation of this response did not appear to be a reflection of desensitization. This result also gave valuable information regarding the concentration of FMRFamide reaching the neurone following an ionophoretic pulse.

Apart from the obvious difference in ionic mechanisms, two other differences exist between the increase in gNa and the increase in gK. The increase in gNa was rapidly desensitized, while the increase in gK does not appear to become desensitized, also the threshold

Fig.3:40. Dose response relationship for the FMRFamide activation of the increase in gNa response. A, relationship between response amplitude (the ordinate is the magnitude of the response and is plotted as positive) and concentration of FMRFamide applied to an F2 neurone voltage clamped at -70 mV. B, ionophoretic application of FMRFamide (▼) in conjunction with bath application showed that subthreshold concentrations of FMRFamide did not produce desensitization of the response.


A
4
2
0
response magnitude (nA)
-7
-5
-3
log FMRFamide conc.
B
1×10-7 M
Fa
1×10-5 M
Fa
1nA
30s
5 × 10-4 M
Fa
2.5nA
30s

for the activation of the increase in gNa was about one hundred times higher than that for the increase in gK. Both of these differences may be interpreted as the existence of different receptors associated with the mediation of the increase in gNa and the increase in gK.

3.10 CHANGES INDUCED BY N-TERMINAL EXTENSIONS.

3.10.1 THE EFFECTS OF THE N-TERMINAL EXTENDED PEPTIDES ON IONIC CURRENTS.

The substitution of Met^2 of FMRFamide for either Leu or Ile had little effect on the responses observed or on the potency of the analogues at producing these responses in Helix neurones. N-terminal extensions of either FMRFamide or FLRFamide, however, had a marked effect on both potency and the type of responses seen (Cottrell and Davies, 1985). Most of the results in this section were obtained using YGGFMRFamide, which is a potent analogue of FMRFamide on many molluscan muscles (Greenberg, Painter and Price, 1981), and pQDPFLRFamide. Recently, pQDPFLRFamide was identified as one of the FMRFamide peptides present in the ganglia of Helix (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman and Riehm, 1985). The N-terminal extended peptides could not be ionophoresed as well as the tetrapeptides, therefore local application onto single neurones was accomplished by pressure ejection (see Methods).

Many neurones were hyperpolarized by YGGFMRFamide and pQDPFLRFamide, some of these responses resembling the increase in gK induced by the tetrapeptides. Like the K response of FMRFamide, altering the external K concentration produced a concomitant shift in the reversal potential of a YGGFMRFamide response recorded from a right parietal neurone, a shift of 26 mV occurring on raising external K concentration from 5 to 15 mM (n=1, Fig.3:41). This was close to the theoretical value of 28 mV for a pure K response.

Many of the hyperpolarizing responses produced by the N-terminal extended peptides were different to those induced by FMRFamide, being much more rapid (Fig.3:42). These responses were recorded as fast outward currents under voltage clamp conditions. A comparison of the rate of decay of the fast response with that of the FMRFamide induced increase in gK is shown in Fig.3:43. The rate of decay was constant from one cell to another, and also from one agonist to another. The decay appeared to fit a single exponential with a time constant of 4 s. The reversal potential of the fast response was close to E_K (see Fig.3:49).

Fig.3:41. The effect of changing the external K concentration on the increase in gK induced by YGGFMRFamide in a neurone in the right parietal ganglion. In control solution (●, 5 mM K), the YGGFMRFamide induced current was reduced to zero when the holding potential was -80 mV. Increasing the K concentration to 15 mM resulted in a reversal of the response at -55 mV.

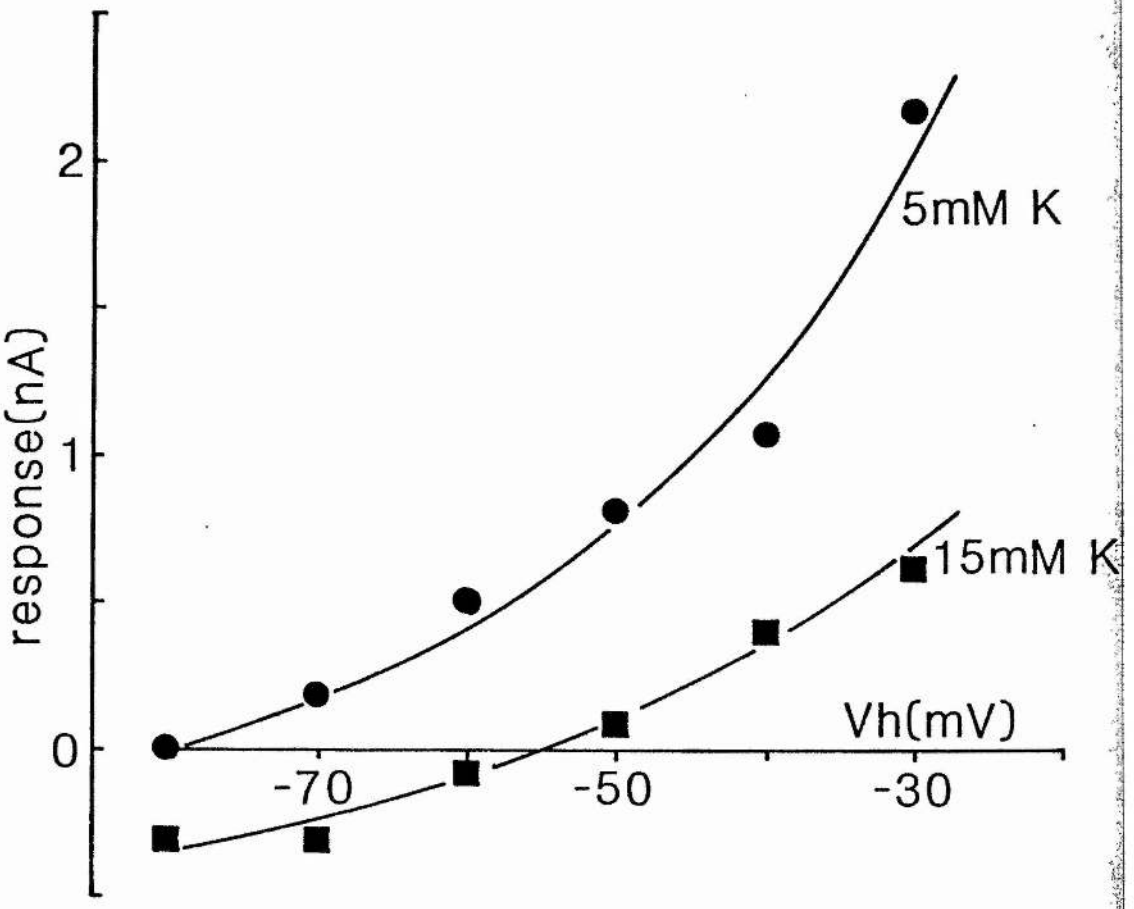
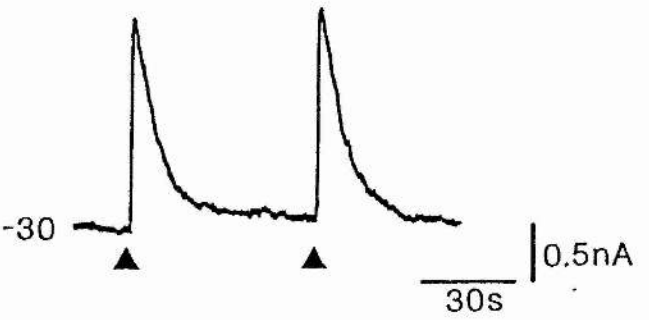
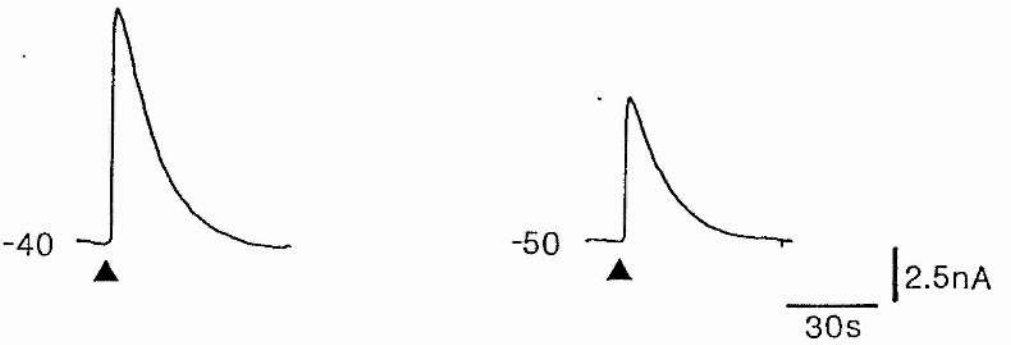

response(nA)
2
1
0
5mM K
15mM K
Vh(mV)
-70
-50
-30

Fig.3:42. Comparison of the fast increase in gK induced by local application of pQDPFLRFamide (▲) in two neurones (top and middle traces) with the slow increase in gK activated by local application of FMRFamide (●) onto a C1 neurone. The holding potential in mV is indicated by the side of each record. The downward deflections on the FMRFamide response at -40 mV are unclamped axon spikes.


-30
0.5nA
30s


-40
-50
2.5nA
30s

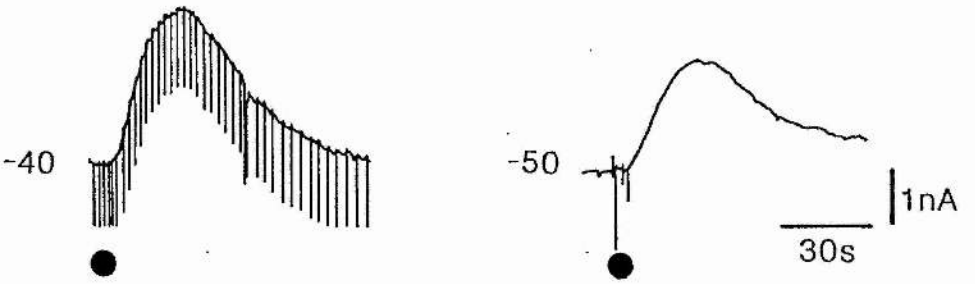
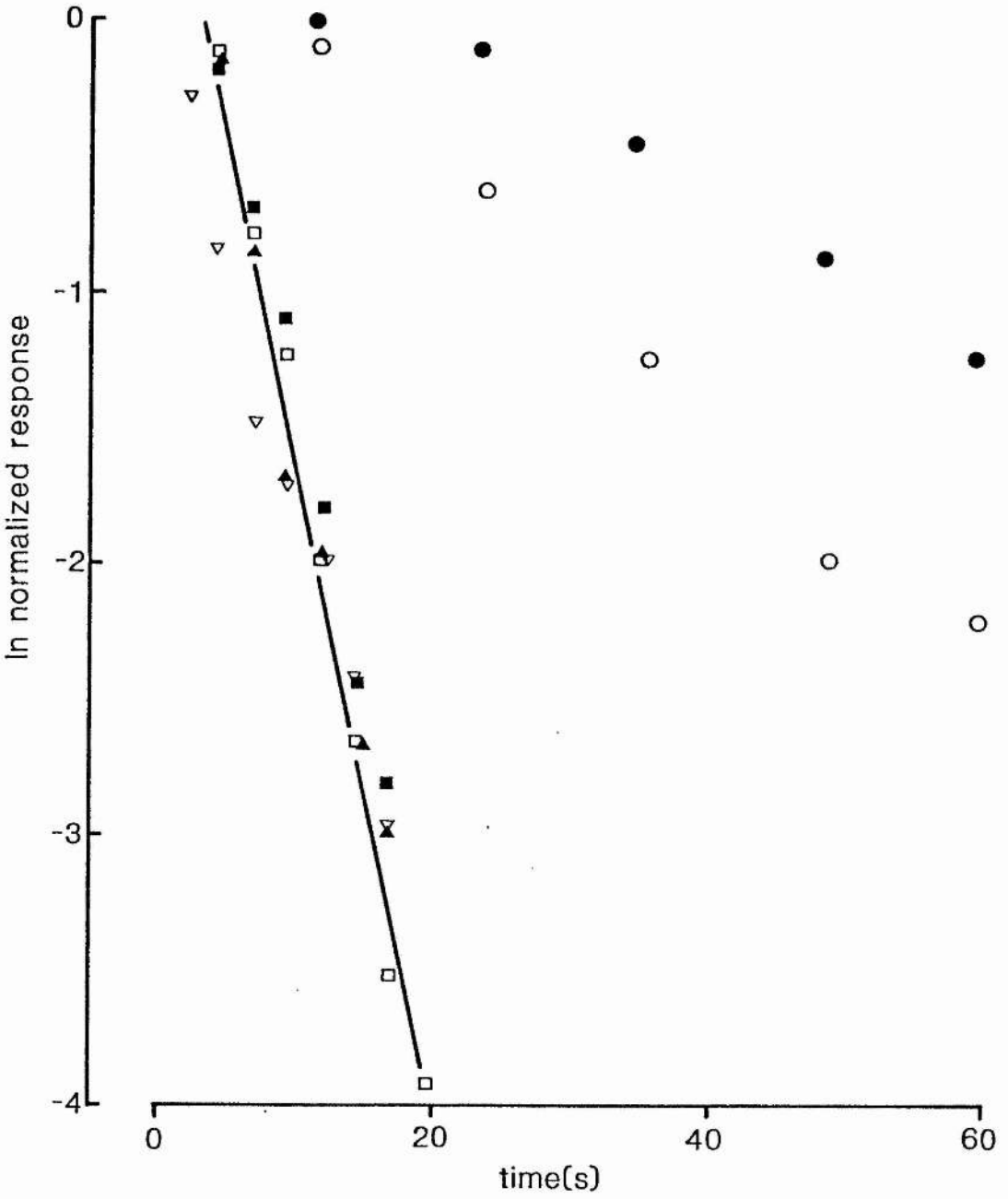

-40
-50
1nA
30s

Fig.3:43. The rate of decay of the fast increase in gK induced by GDPFLRFamide (■) and PFLRFamide (□) (see later) in one neurone, and by pQDPFLRFamide (▲,▽) in two other neurones, compared with the decay of two slow increases in gK induced by FMRFamide in different neurones (●,○,). The ordinate is the natural log of the currents normalized to the maximum amplitude of the respective responses. The rate of decay of the slow increase in gK is variable, but the decay of the fast increase in gK induced by different N-terminal extended peptides, even in different neurones, is remarkably similar.


0
-1
-2
-3
-4
ln normalized response
0
20
40
60
time(s)

3.10.2 THE EFFECT OF K CHANNEL BLOCKERS ON THE FAST HYPERPOLARIZATION.

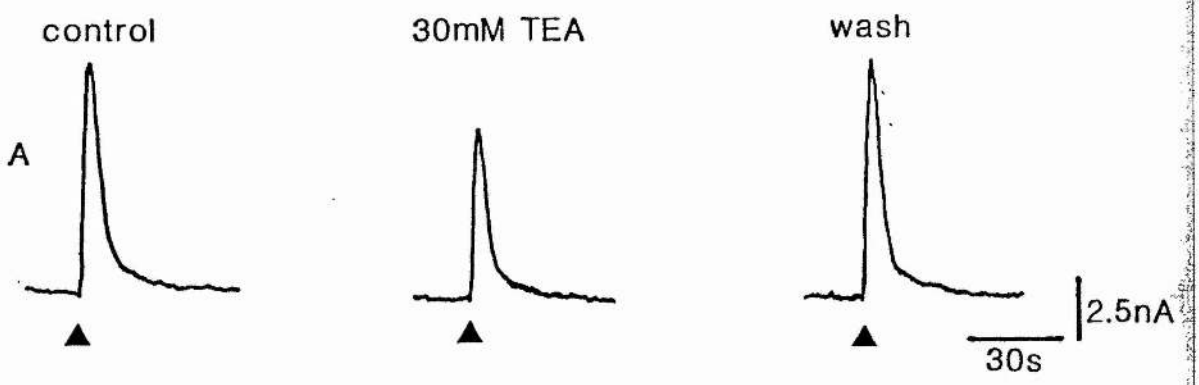
The fast hyperpolarization was sensitive to the K current blockers TEA and Cs^+ ions. 30 mM TEA reduced the amplitude of the fast outward current elicited by pQDPFLRFamide (n=2, Fig.3:44A). The response was less sensitive to TEA than the FMRFamide induced increase in gK (c.f. Fig.3:9). Intracellular Cs^+ ions markedly attenuated the response. The amplitude of the response of a neurone in the right parietal ganglion to pQDPFLRFamide fell by 80% after 12 minutes following impalement with a recording electrode filled with CsCl (n=1, Fig.3:44B). These results, together with the reversal potential and voltage dependency are consistent with the fast hyperpolarization being mediated by a fast increase in gK.

The heptapeptides activated both fast and slow increases in gK, although they were less potent than FMRFamide at producing the slow increase in gK (see page 104). Activation of a fast increase in gK by FMRFamide was not observed. Further evidence that these two K responses were different, and not merely an artefact of concentration, was indicated by two experiments. The first involved the response of an unidentified neurone in the right parietal ganglion to pQDPFLRFamide (Fig.3:45).

This neurone responded to application of pQDPFLRFamide with a combination of a fast and slow increase in gK. Altering the position of the pQDPFLRFamide micropipette changed the relative components of the response. This suggested that there may be a differential distribution on the neurone of the receptor-ionophore complexes producing the responses as was found for ACh in Navanax by Levitan and Tauc (1972).

A second experiment consisted of observing the effect of increasing the distance of the pQDPFLRFamide pipette on the amplitude and time course of the fast increase in gK. The result of such an experiment is shown in Fig.3:4b. As the peptide pipette is moved away, the amplitude of the response decreased, although the time course of the response was unaltered. There was also an increasing delay between the pressure pulse and the onset of the response as the distance of the pQDPFLRFamide pipette from the cell was increased. Thus two hyperpolarizing responses were induced by FMRFamide peptides; a fast increase in gK and a slow increase in gK.

Fig.3:44. The effect of TEA and intracellular Cs on the fast increase in gK induced by pQDPFLRFamide in different neurones in the right parietal ganglion. Both neurones were voltage clamped at -40 mV. A, exposure of the preparation to solution containing 30 mM TEA slightly reduced the response to locally applied pQDPFLRFamide (▲). The response recovered after washing with control solution. B, impalement of a neurone with a recording electrode filled with 2 M CsCl resulted in a marked reduction with time in the amplitude of the pQDPFLRFamide induced fast increase in gK.


control
30mM TEA
wash
A
2.5nA
30s

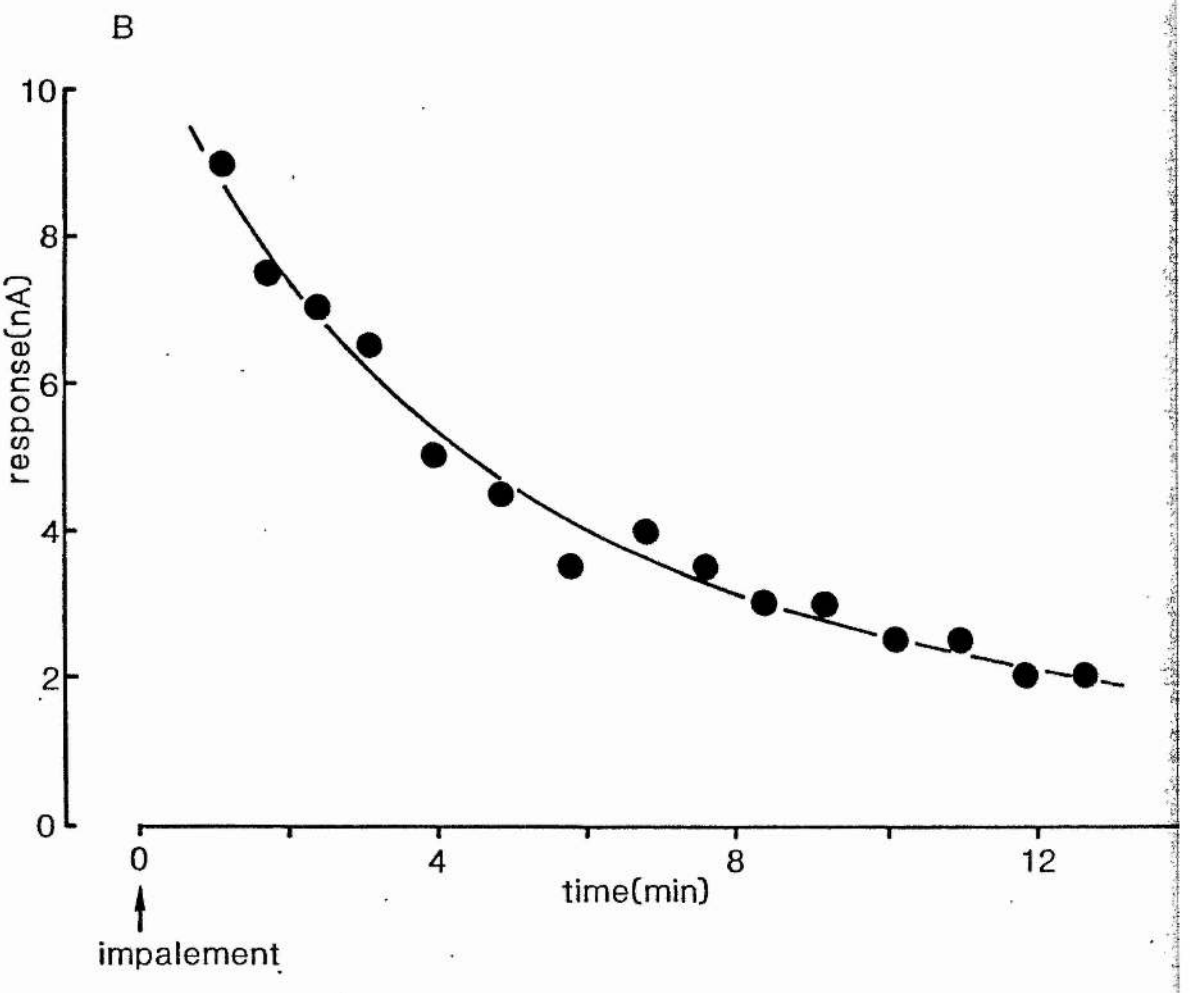

B
10
8
6
4
2
0
response(nA)
0
4
8
12
time(min)
impalement

Fig.3:45. Application of pQDPFLRFamide to a neurone in the right parietal ganglion evoked a response which was a combination of slow and fast increases in gK. The neurone was voltage clamped at -30 mV and pQDPFLRFamide was applied by pressure ejection from a micropipette (▲). Each trace shows the response observed after placing the peptide pipette in different positions around the soma of the cell.

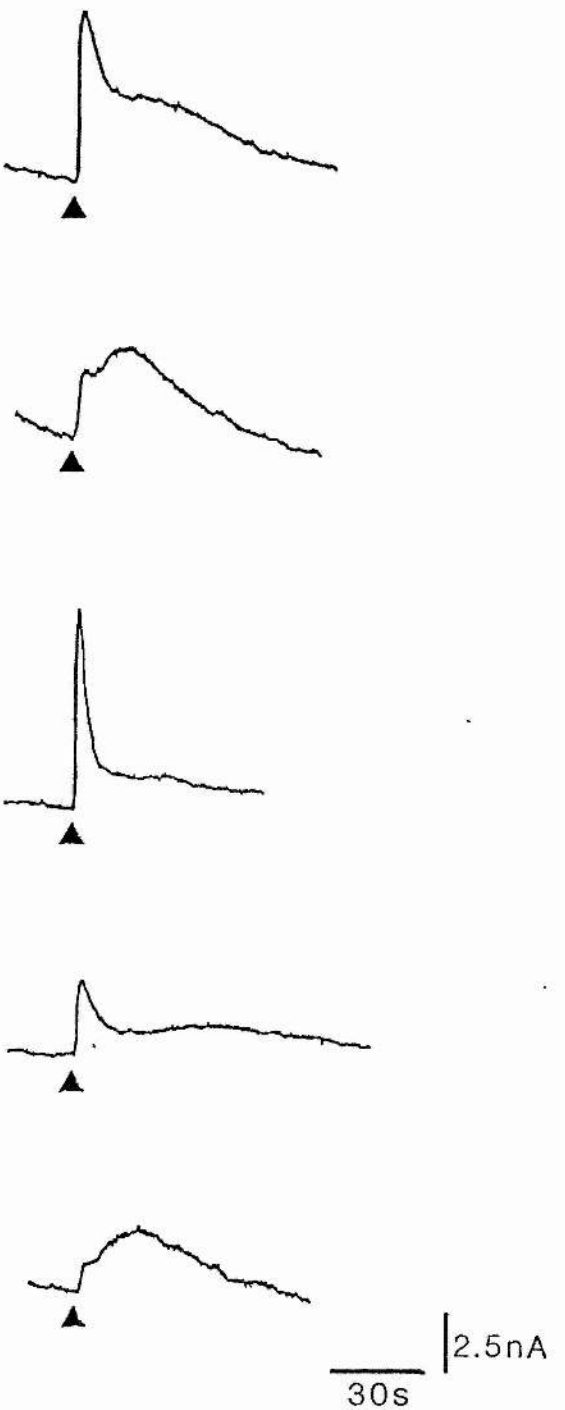
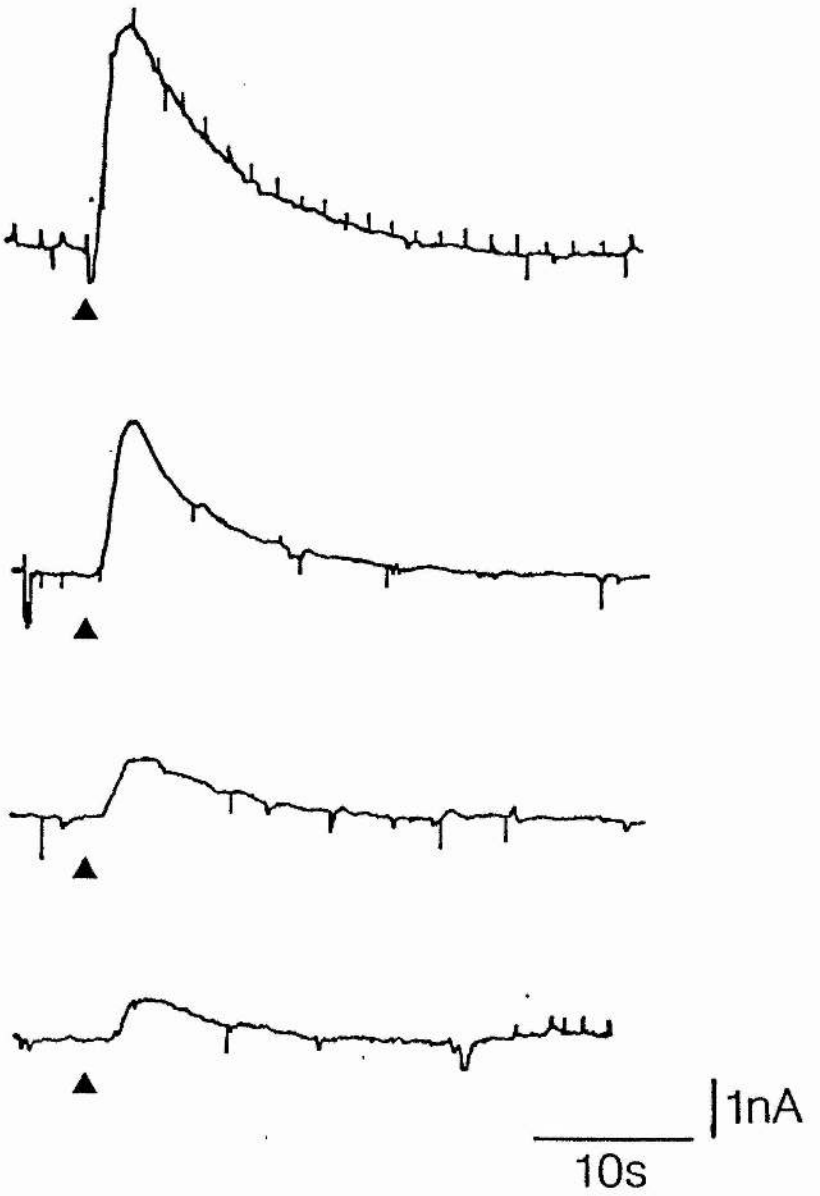

2.5nA
30s

Fig.3:46. A neurone in the right parietal ganglion responding to pQDPFLRFamide with a fast increase in gK was voltage clamped at -40 mV. The peptide was applied by pressure ejection (▲). On moving the peptide pipette further away from the neurone in small steps, the amplitude of the response decreased, although the time course remained similar. The delay between peptide application and the onset of the response increased accordingly, thus the response does not appear to be a pressure artefact. The inward deflection in the top recording immediately following peptide application is probably a pressure artefact.


1nA
10s

3.10.3 ACTIVATION OF THE VOLTAGE DEPENDENT RESPONSE IN THE C1 NEURONE BY pQDPFLRFamide.

YGGFMRFamide has been reported to be more potent than FMRFamide at producing the voltage dependent response in the C1 neurone. Application of pQDPFLRFamide onto this neurone also induced the voltage dependent response (Davies, 1984). The relation between response amplitude and membrane potential was similar to that of the YGGFMRFamide and 5-HT responses (Fig.3:47). Like YGGFMRFamide, pQDPFLRFamide was more potent than FMRFamide at producing this response.

3.10.4 THE N-TERMINAL EXTENDED PEPTIDES DID NOT ACTIVATE THE INCREASE IN gNa.

The increase in gNa response was present in both E13 and F2 neurones. Local application of YGGFMRFamide, pQDPFLRFamide and PFLRFamide onto these neurones did not produce an increase in gNa (n=15, Fig.3:48). This result using pressure ejection of the peptides was confirmed by the failure of bath application of 1 mM YGGFMRFamide to elicit this response (n=1).

Fig.3:47. Activation of the voltage dependent decrease in gK by pQDPFLRFamide in a voltage clamped C1 neurone. The response was absent at -45 mV, but increased markedly with depolarization. The inset shows an example of the current induced at a holding potential of -30 mV.

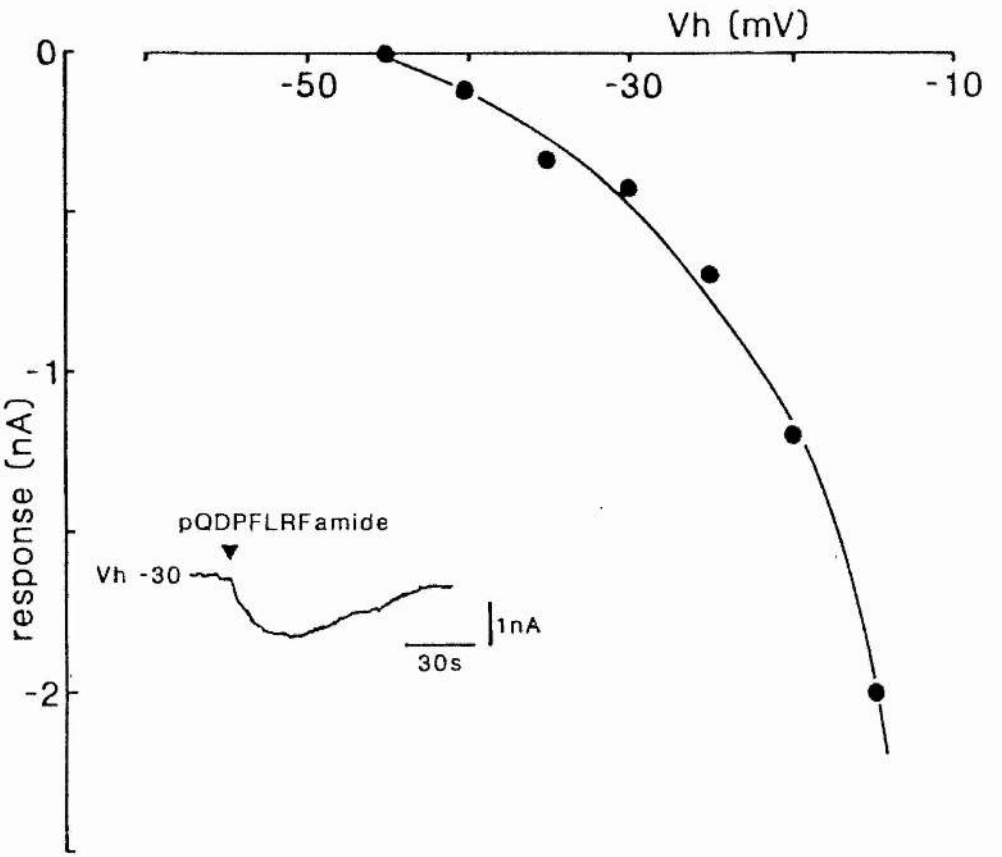
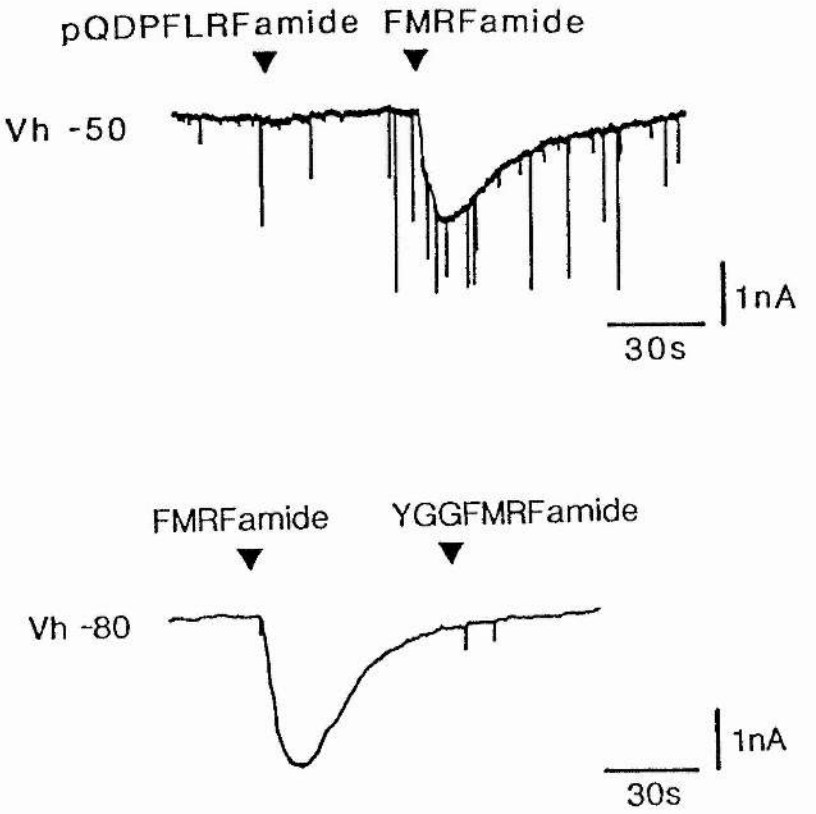

Vh (mV)
0
-50
-30
-10
-1
-2
response (nA)
pQDPFLRFamide
Vh -30
1nA
30s

Fig.3:48. Application of pQDPFLRFamide to an F2 neurone (top) and YGGFMRFamide to an E13 neurone (bottom) failed to activate the increase in gNa, which was evoked by FMRFamide in both of these neurones. The peptide pipettes were placed in several positions to account for the possibility of a localized distribution of receptors. The reason for the failure of pQDPFLRFamide to activate an increase in gK in the F2 neurone is not known. This result was confirmed by bath application (see text).


pQDPFLRFamide
FMRFamide
Vh -50
1nA
30s
FMRFamide
YGGFMRFamide
Vh -80
1nA
30s

The responses observed with the N-terminal extended peptides were very different to those induced by the tetrapeptides. The main differences were the lack of activation of the increase in gNa, and the production of a fast increase in gK by the longer chain peptides. The next section describes the results obtained when the effect of the N-terminal extended peptides were compared with the effects of FMRFamide and FLRFamide on C1 and F2 neurones.

3.11 COMPARISON OF THE RESPONSE OF THE F2 NEURONE TO FMRFamide, YGGFMRFamide AND pQDPFLRFamide.

The increase in both gNa and gK induced by FMRFamide in the F2 neurone provided a useful system for comparing the actions of FMRFamide with those of the extended peptides. The effects of the peptides were compared by local application onto voltage clamped F2 neurones. A comparison of the effects was often made over a range of holding potentials.

The relationship between response amplitude and holding potential for the effects of FMRFamide and YGGFMRFamide on an F2 neurone is shown in Fig.3:49A. The FMRFamide response clearly reversed between -40 and -50 mV. However, the YGGFMRFamide response was an outward current at all potentials less negative than -80 mV. An almost identical result was obtained when the effects of pQDPFLRFamide and FMRFamide were compared in a different F2 neurone, although in this neurone, the reversal potential of the FMRFamide response was more positive (Fig.3:49B). The reversal potential of the pQDPFLRFamide response was -80 mV (the same potential at which the YGGFMRFamide induced current was likely to reverse in the experiment shown in Fig.3:49A). The response of this F2 neurone to YGGFMRFamide (obtained at two holding potentials only) was very similar to the pQDPFLRFamide

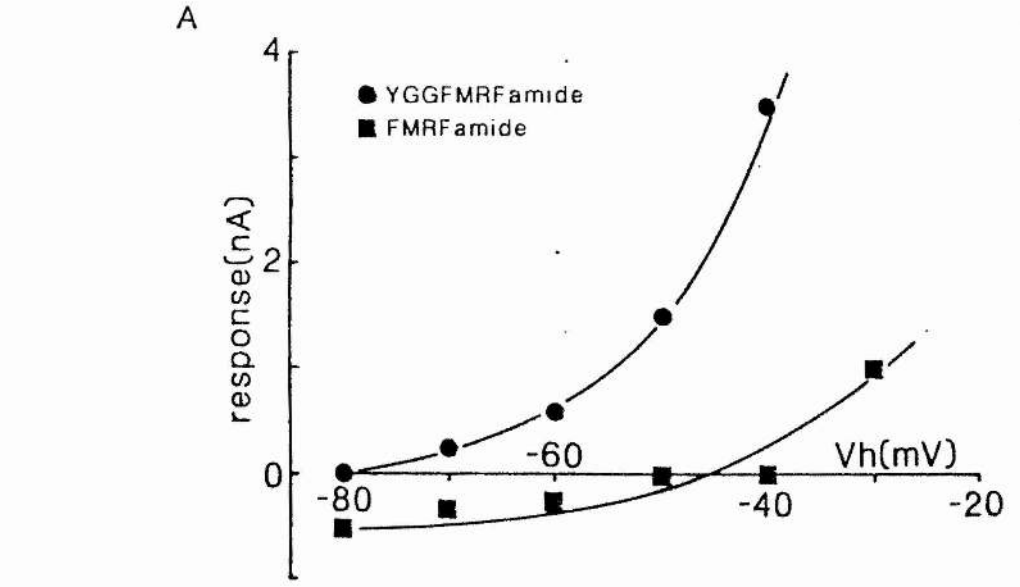
response, both having the characteristics of the fast increase in gK (Fig.3:50).

Responses induced in the F2 neurone by YGGFMRFamide and pQDPFLRFamide were therefore quite different to those induced by FMRFamide. The differences observed were consistent with a lack of activation of the increase in gNa by YGGFMRFamide and pQDPFLRFamide, which was also evident in E13. Thus the F2 neurone responded to these two peptides with an increase in gK only.

3.12 THE EFFECTS OF PFLRFamide RESEMBLE THOSE OF THE HEPTAPEPTIDES.

Extending the N-terminal of FMRFamide or FLRFamide by three amino acids produced marked alterations in biological activity. These included a lack of activation of the increase in gNa response and an apparent increase in potency at producing the voltage dependent response. It was therefore interesting to compare the effects of a single amino acid extension of the N-terminal with those of the tetrapeptides. The peptide used in this investigation was PFLRFamide, which was compared with FLRFamide in order to keep the structure as close as possible.

Fig.3:49. A, comparison of the response of an F2 neurone to local application of FMRFamide and YGGFMRFamide. B, comparison of the response of another F2 neurone to locally applied FMRFamide and pQDPFLRFamide. Both F2 neurones were voltage clamped. The amplitudes of the responses are plotted against holding potential.


A
4
2
0
response(nA)
YGGFMRFamide
FMRFamide
-60
Vh(mV)
-80
-40
-20

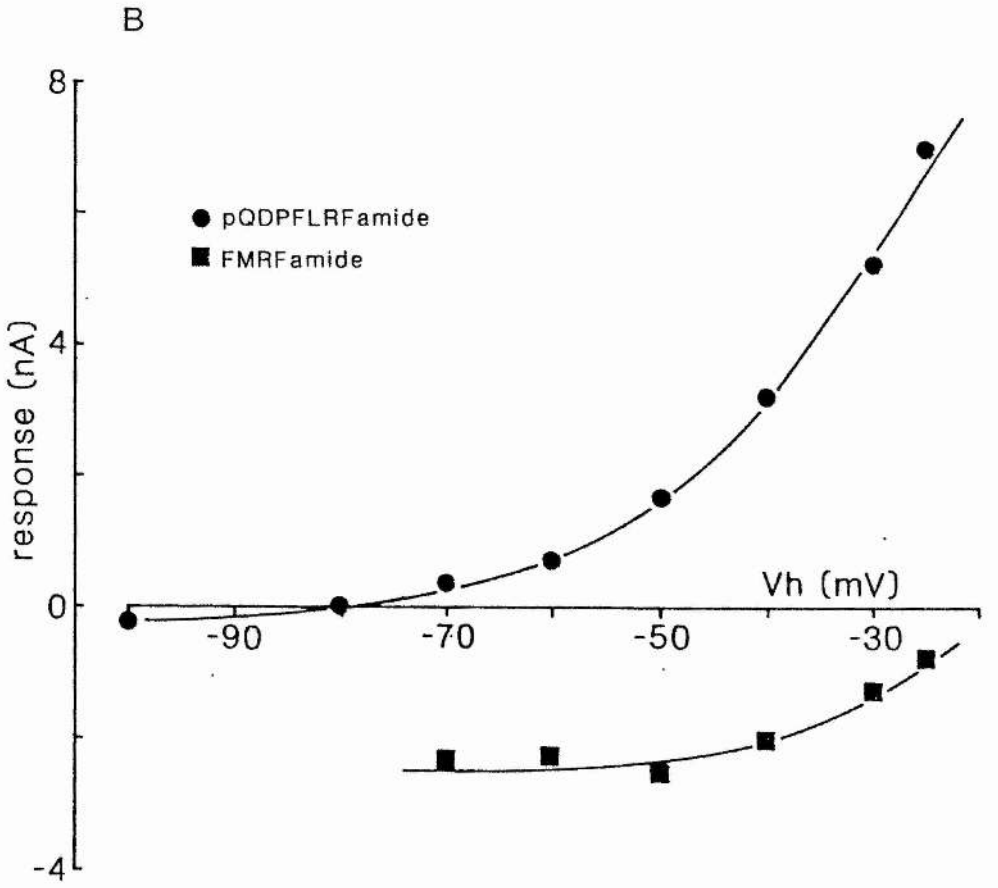
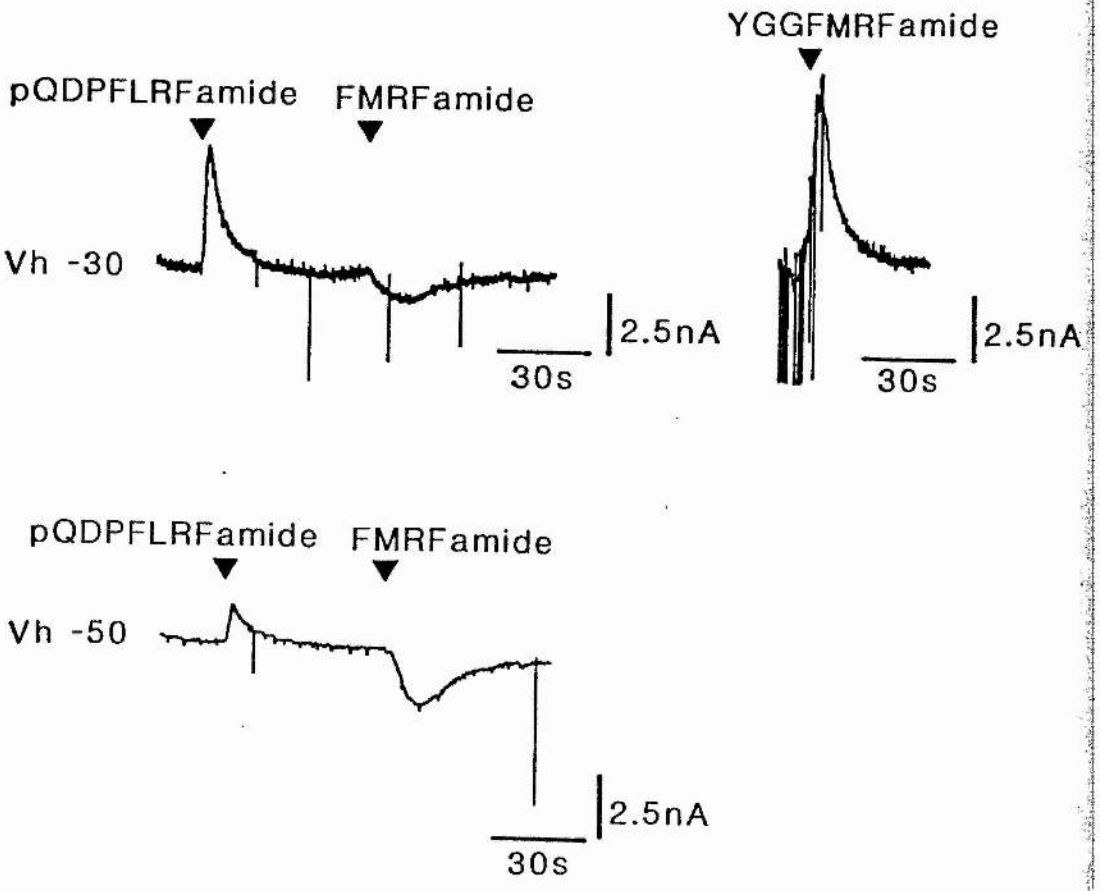

B
8
4
0
-4
response (nA)
pQDPFLRFamide
FMRFamide
Vh (mV)
-90
-70
-50
-30

Fig.3:50. Recordings of the responses obtained from the F2 neurone of Fig.3:49B. Application of both pQDPFLRFamide and YGGFMRFamide onto this neurone produced a fast increase in gK. The current response to FMRFamide was inward at both potentials shown. Vh, holding potential (mV).


pQDPFLRFamide
FMRFamide
Vh -30
2.5nA
30s
YGGFMRFamide
2.5nA
30s
pQDPFLRFamide
FMRFamide
Vh -50
2.5nA
30s

Both FLRFamide and PFLRFamide were applied by pressure ejection onto individual neurones, or by advancing a 'leaky' micropipette close to the cell. Advancing a leaky FLRFamide containing pipette close to an F2 neurone voltage clamped at -80 mV produced an inward current as the result of the activation of the increase in gNa. A similar procedure using a pipette containing PFLRFamide had no effect, suggesting that PFLRFamide, like the heptapeptides did not activate the increase in gNa. A similar procedure performed when the holding potential was at -50 mV resulted in both peptides inducing an outward K current (n=2, Fig.3:51A). This acted as a control to ensure that a sufficient concentration of both peptides was reaching the neurone. No interaction between FLRFamide and PFLRFamide on the F2 neurone was seen. Application of FLRFamide caused an increase in gNa, which remained unaltered during simultaneous application of PFLRFamide (Fig.3:51B). Therefore PFLRFamide did not antagonise FLRFamide, neither did it cause a desensitization of the FLRFamide response.

Pressure ejection of PFLRFamide onto an unidentified neurone in the right parietal ganglion produced an increase in gK. The relationship between membrane potential and response amplitude resembled that for the FMRFamide and pQDPFLRFamide K responses (Fig.3:52). Application of PFLRFamide onto another neurone induced a

Fig.3:51. Comparison of the effect of FLRFamide and PFLRFamide on an F2 neurone. A, advancing leaky pipettes containing FLRFamide (▲) and PFLRFamide (●) towards a voltage clamped F2 neurone. At a holding potential of -50 mV, both peptides produce an increase in gK. At -80 mV, FLRFamide induced an increase in gNa, but no response was induced by PFLRFamide. B, the increase in gNa induced by FLRFamide (▼) was unaffected by the application of PFLRFamide (solid line). The concentration of peptides in the pipettes was 1 mM.

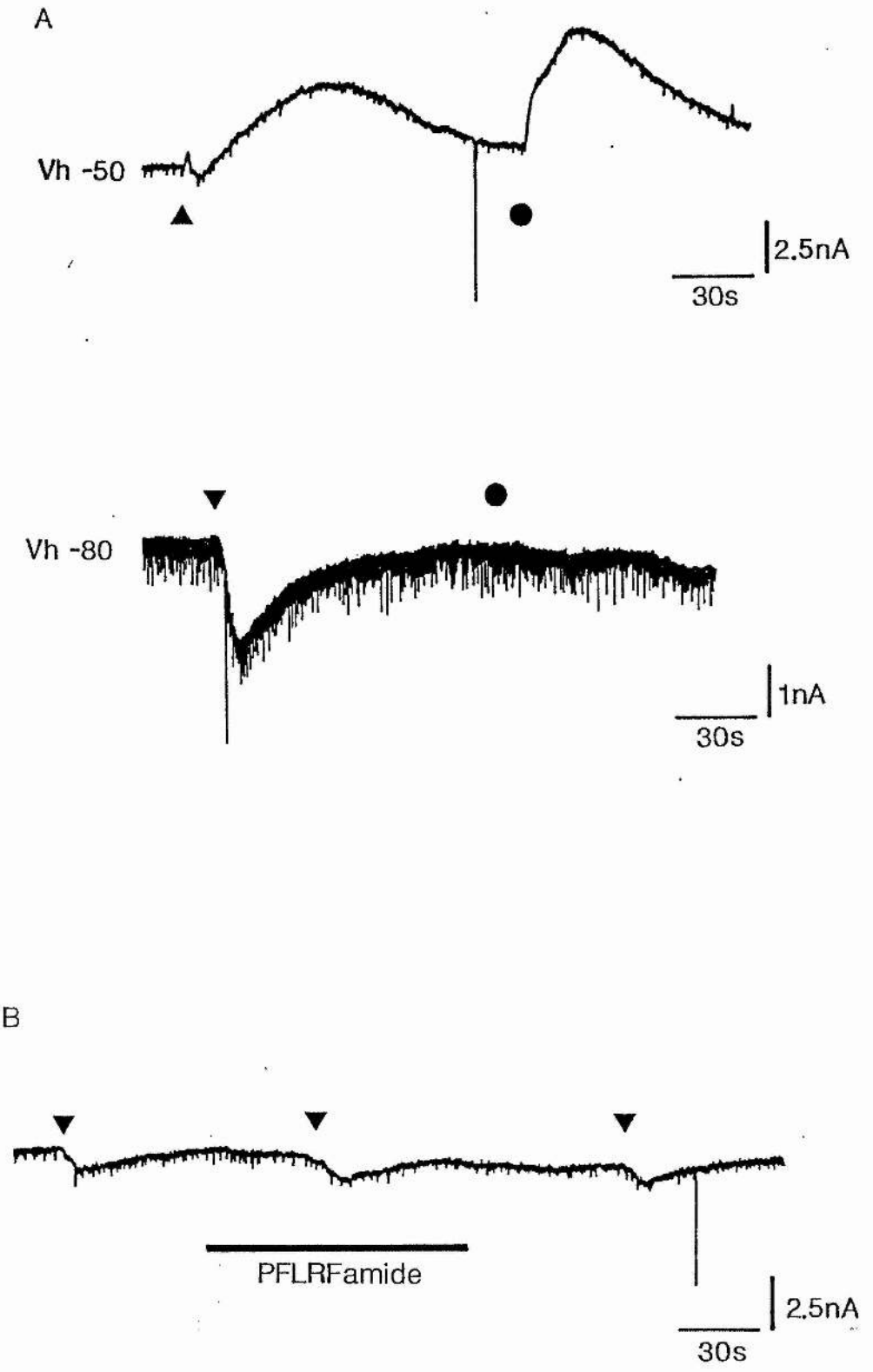
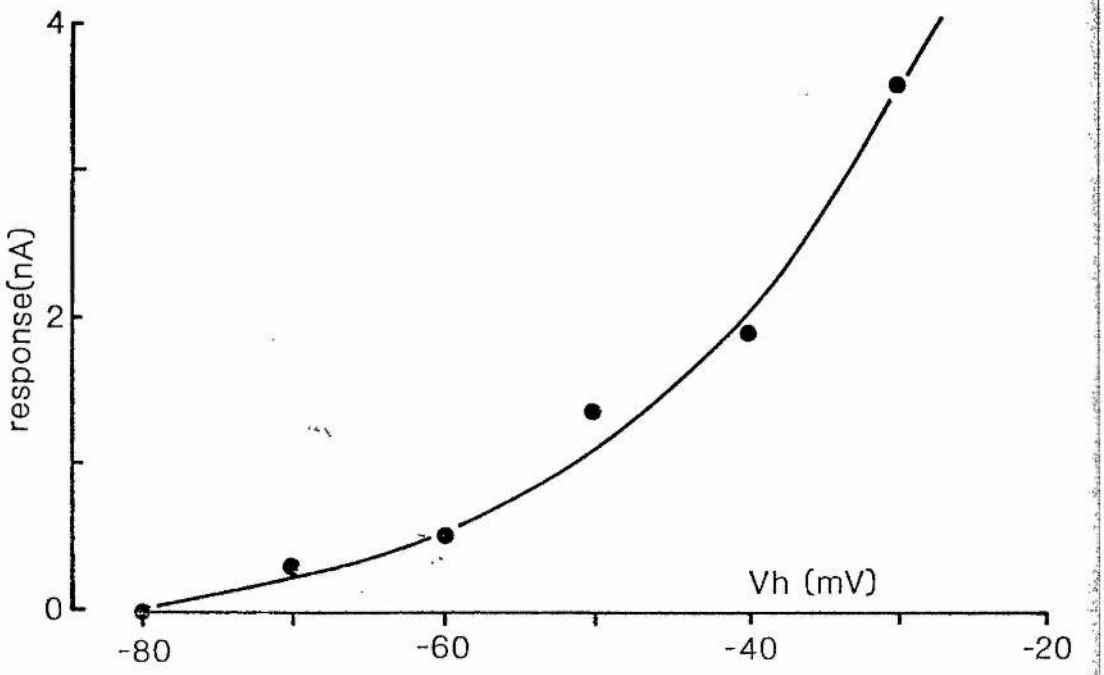

A
Vh -50
2.5nA
30s
Vh -80
1nA
30s
B
PFLRFamide
2.5nA
30s

Fig.3:52. Relationship between holding potential and the amplitude of the PFLRFamide induced outward current in an unidentified neurone in the right parietal ganglion. The voltage dependency is very similar to the increase in gK induced by the other peptides (c.f. Fig.3:49.).


4
2
0
response(nA)
Vh (mV)
-80
-60
-40
-20

fast increase in gK. Interestingly GDPFLRFamide also produced a fast increase in gK in the same neurone. These results show that the effects induced by PFLRFamide resemble those induced by the heptapeptides. Thus it seems that extending the N-terminal by one proline alters the biological activity quite markedly.

3.13 COMPARISON OF THE RESPONSE OF THE C1 NEURONE TO FMRFamide, YGGFMRFamide AND pQDPFLRFamide.

FMRFamide reliably produced an increase in gK in the C1 neurone. This increase in gK had a tendency to mask the decrease in gK which also occurred in this neurone. Occasionally both YGGFMRFamide and pQDPFLRFamide induced an increase in gK in the C1 neurone. When the increase in gK was observed it was similar to the FMRFamide induced increase in gK (Fig.3:53A). A fast K response was not observed in the C1 neurone. Both YGGFMRFamide and pQDPFLRFamide activated the decrease in gK in preference to producing an increase in gK (Fig.3:53B). Infact a substantial slow increase in gK was rarely induced by the heptapeptides.

There are many possible interpretations of the results obtained from the C1 neurone. It could be that the heptapeptides were more potent at producing the decrease in gK, less potent at producing an increase in gK, or a combination of these two possibilities. Because

of the difficulty in separating the voltage dependent response from the increase in gK, it was difficult to obtain relative potencies of the peptides at inducing the voltage dependent response. However, a dose response relationship for the YGGFMRFamide activation of the increase in gK was obtained by voltage clamping a C1 neurone at -45 mV, a potential at which the voltage dependent response was not activated. The relationship obtained, compared with the relationship for the FMRFamide activation of the response in the same neurone, is shown in Fig.3:54 (n=1). The maximal response elicited by YGGFMRFamide was much smaller than that induced by FMRFamide. In another C1 neurone, 1×10^{-4} M pQDPFLRFamide did not produce a substantial increase in gK.

The greater potency of the heptapeptides at producing the decrease in gK could therefore be explained by their lower potency towards activating the slow increase in gK. However, it is not known whether there is a difference in potency between these N-terminal extended peptides and the tetrapeptides towards inducing the voltage dependent decrease in gK.

Fig.3:53. A, both FMRFamide and pQDPFLRFamide produced a similar slow increase in gK when applied to a C1 neurone voltage clamped at -40 mV. In a different C1 neurone, YGGFMRFamide also produced a slow increase in gK. B, different responses induced in a C1 neurone by application of FMRFamide and pQDPFLRFamide. FMRFamide produced mainly a slow increase in gK, while pQDPFLRFamide induced the voltage dependent response only. Vh, holding potential.

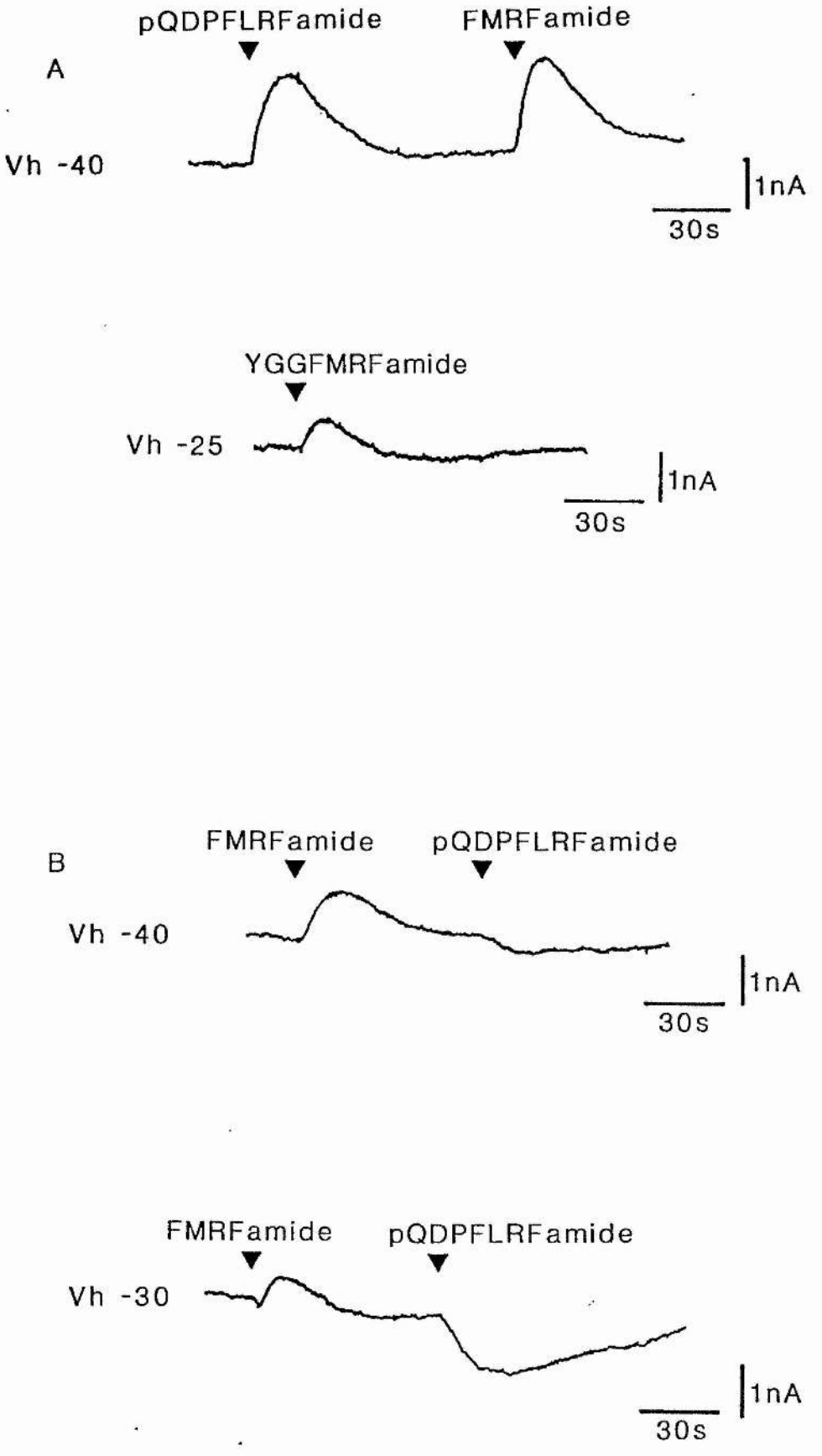
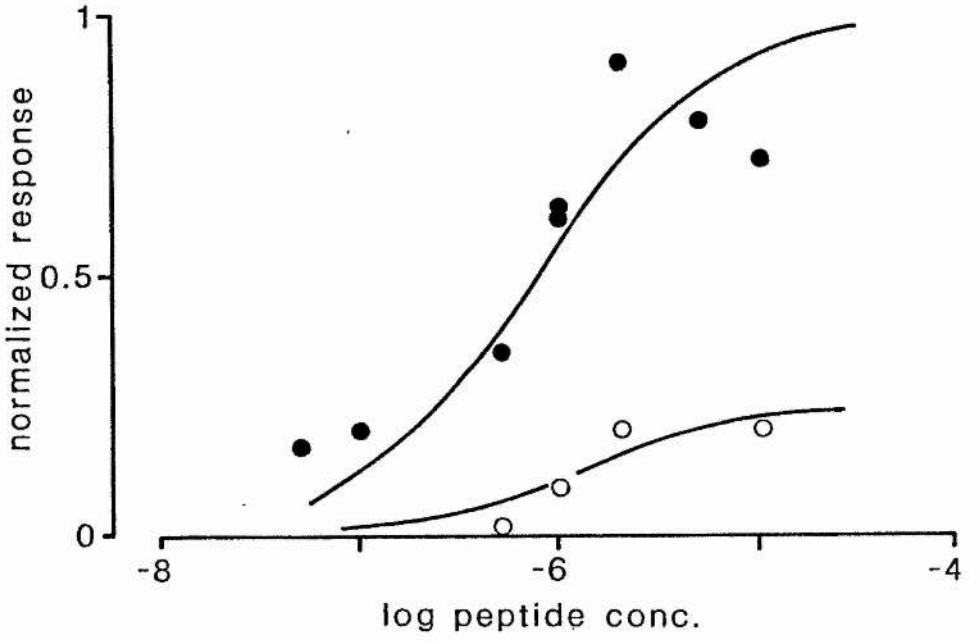

pQDPFLRFamide
FMRFamide
A
Vh -40
1nA
30s
YGGFMRFamide
Vh -25
1nA
30s
FMRFamide
pQDPFLRFamide
B
Vh -40
1nA
30s
FMRFamide
pQDPFLRFamide
Vh -30
1nA
30s

Fig.3:54. Comparison of the activation of the increase in gK by FMRFamide and YGGFMRFamide in a C1 neurone voltage clamped at -45 mV. The responses were normalized to the estimated maximum FMRFamide response. (●) FMRFamide, (○) YGGFMRFamide. The solid lines are a plot of:

$$N = C/(C + Kd)$$

where N is the normalized response, C is the concentration of peptide and Kd is the dissociation constant. The Kd values used were 8×10^{-7} for FMRFamide and 1.2×10^{-6} for YGGFMRFamide.


normalized response
1
0.5
0
-8
-6
-4
log peptide conc.

3.14 COMPARISON OF THE ACTIONS OF NON-FMRFamide PEPTIDES WITH THOSE OF FMRFamide.

The specificity of the actions of FMRFamide peptides was tested by the application of various other peptides to neurones which responded to FMRFamide. The non-FMRFamide peptides tested were:

YGGFMRF

YGGFM

pQDPFLRLamide

pQDPFLRIamide

These peptides were applied locally to the C1 neurone, the F2 neurone and a number of other unidentified neurones. Neither pQDPFLRLamide nor pQDPFLRIamide induced responses when tested on the C1 and F2 neurones over a range of holding potentials (n=2). In an unidentified cell in the right parietal ganglion, application of both pQDPFLRLamide and pQDPFLRIamide failed to produce a response at a concentration of 1×10^{-4} M. The response to pQDPFLRFamide in this neurone was a fast increase in gK.

Locally applied YGGFM (met-enkephalin) to the C1 neurone produced a voltage dependent inward current (n=4), however, the potency of YGGFM was lower than that of YGGFMRFamide at producing this response. No other

FMRFamide-like responses were observed with YGGFM. No responses were induced by YGGFMRF on either the C1 or the F2 neurone.

3.15 SUMMARY OF THE RESULTS.

The actions of FMRFamide and some FMRFamide analogues on Helix neurones were investigated using electrophysiological techniques. FMRFamide induced three different responses, two involving an increase in conductance and one the result of a decrease in conductance. The three responses observed were:

(1) increase in gK
(2) increase in gNa
(3) decrease in gK

The ionic mechanism underlying the decrease in gK was more difficult to study than the other two, however it appeared to be a decrease in a K current which may be sensitive to cyclic nucleotides.

Interesting effects were observed when FMRFamide analogues were tested. The tetrapeptides behaved very much like FMRFamide, but the N-terminal extended peptides behaved differently. The major difference was the lack of activation of the increase in gNa and the activation of a fourth response, a fast increase in gK, by the extended peptides. The FMRFamide peptides could therefore be divided into two groups according to their biological activity, they are the tetrapeptides which activate mainly

an increase in gNa and a slow increase in gK, and the N-terminal extended peptides which activate mainly a fast increase in gK and a voltage dependent decrease in gK.

CHAPTER 4

DISCUSSION

4.1 ACTIONS OF FMRFamide.

FMRFamide exerted three ionic actions on Helix neurones. Two of these were the result of an increase in ionic conductance and the third was the result of a decrease in ionic conductance. Many other compounds induce multiple actions on molluscan neurones, for example ACh (Kehoe, 1972a), 5-HT (Gerschenfeld and Paupardin-Tritsch, 1974) and dopamine (Ascher, 1972; Berry and Cottrell, 1979). In some cases the diversity of effects produced by a single neurotransmitter may be attributed to the existence of multiple receptor sites for that compound. Kehoe (1972b) reported that the three ACh responses in Aplysia are mediated by the activation of three distinct receptors. Multiple receptor sites have also been found for 5-HT (Gerschenfeld and Paupardin-Tritsch, 1974), histamine (Gruol and Weinrich, 1980) and many other compounds (Ascher and Kehoe, 1975).

In many respects, FMRFamide exhibits 'transmitter-like' effects on Helix neurones. These include the increase in gK and increase in gNa responses, which cause a hyperpolarization and depolarization of the neurones exhibiting these responses. On the other hand, the voltage dependent decrease in gK, induced mainly by N-terminal extended analogues, being absent at resting membrane potentials, implies a neuromodulatory action by these peptides. The actions of FMRFamide and related

analogues on ionic conductances, together with implications for the existence of multiple receptor sites will be discussed in detail in this chapter.

4.2 THE HYPERPOLARIZING RESPONSE INDUCED BY FMRFamide.

4.2.1 THE IONIC MECHANISM OF THE HYPERPOLARIZATION.

Two responses resulting from an increase in ionic conductance were induced by application of FMRFamide onto individual neurones. One response was a hyperpolarization, which was observed as an outward current under voltage clamp conditions. The other was a depolarization, which was observed as an inward current during voltage clamp.

All hyperpolarizing responses, whatever their particular mechanism, result in an increase in the net amount of negative charge within a cell. In the case of the FMRFamide induced hyperpolarization of snail neurones, this was accomplished by an outward movement of K^+ ions. This was observed as an outward current during voltage clamp, which could be reversed to an inward current by holding the membrane potential more negative than E_K.

The dependence of the response on external K concentration yielded valuable information regarding the specificity of the channels activated by FMRFamide. By definition, E_K is the potential at which the net flow of K^+ ions across the membrane is zero, regardless of the

membrane permeability to K^+ ions. Therefore, if the FMRFamide induced conductance was due to an increase in permeability exclusively to K^+ ions, application of FMRFamide should not cause a change in membrane current if the membrane potential was equal to E_K. Because the internal concentration of K was not known accurately, an absolute value of E_K could not be obtained. Therefore, the change in the reversal potential of the response was compared with the calculated change in E_K following alterations of external K concentration. The reversal potential of the FMRFamide response followed the calculated change in E_K when the external K concentration was altered. Occasionally there was a slight deviation between the magnitude of the calculated change in E_K and the observed change in the reversal potential of the response. This discrepancy could arise from a change in the intracellular concentration of K during the course of the experiment, as was suggested for a carbachol induced increase in gK in Aplysia (Ginsborg and Kado, 1975). The calculation of the change in E_K following alterations of external K concentration assumes a constant intracellular K concentration. If this assumption was relaxed in practice, then the calculated change would deviate from the actual change in E_K.

4.2.2 K CHANNEL BLOCKERS ATTENUATE THE INCREASE IN gK.

Both TEA and Cs^+ ions have been shown to block K currents of molluscan soma (Hermann and Gorman, 1981b; Tillotson and Horn, 1978). TEA markedly reduced the FMRFamide induced K current. The concentration of TEA used (30 mM) was comparable to that shown to block time and voltage dependent K currents in molluscan soma (50 mM Hermann and Gorman, 1981b; 100 mM Meech and Standen, 1975). This is in contrast to the much lower concentration (1 mM) used by Kehoe (1972a) to block ACh responses in Aplysia. In the cholinergic system of Aplysia, TEA is believed to inhibit at the ACh binding site, rather than at the channel level. The reduction of the FMRFamide response by TEA was voltage dependent, being greater at more negative potentials. A calculation using the data of Fig.3:9 gives a reduction of 85% at -50 mV and 68% at -30 mV. A similar voltage dependency for the block of K currents by externally applied TEA, i.e. greater block with increasing hyperpolarization, has been shown by Hermann and Gorman (1981b) This implied that TEA was acting on the channel which is activated during the FMRFamide response, and not on the receptor.

Intracellular loading of molluscan neurones by Cs leads to an almost complete block of K currents. Intracellular Cs^+ ions have been used in this way to block K currents so as to successfully unmask other voltage activated currents in molluscan soma (Akaike, Lee and Brown, 1978; Tillotson and Horn, 1978). The effect of Cs on the FMRFamide response was investigated by injecting Cs^+ ions into the cell from a recording electrode filled with 2 M CsCl solution. Because the neurones were immediately impaled with a Cs containing electrode, no direct control was possible, however the reduction of steady state outward currents progressed with time after impalement, suggesting that the reduction increased with increasing concentration of Cs^+ ions inside the cell. A similar time dependent reduction in the amplitude of the FMRFamide induced K current was observed; the longer the time after impalement, then the greater was the reduction in the amplitude of the response.

This FMRFamide induced increase in gK has been reported to be sensitive to 4-aminopyridine (Boyd and Walker, 1985), which blocks K currents that tend to be rather insensitive to TEA (Thompson, 1977; Hermann and Gorman, 1981a). Cottrell (1982a) reported that cooling the preparation, which suppresses K mediated responses in Aplysia neurones (Kehoe, 1972a; Swann and Carpenter, 1975), also reduced the FMRFamide response.

4.2.3 THE VOLTAGE DEPENDENCE OF THE INCREASE IN gK.

The relationship between the amplitude of the FMRFamide induced K current and membrane potential was non-linear, the FMRFamide induced conductance being greater at depolarized potentials. This rectification displayed by the FMRFamide response was more pronounced than that predicted by the constant field model of Goldman (1943) and Hodgkin and Katz (1949), implying that the behaviour of the channels activated by FMRFamide was dependent in some way on membrane potential. A limited number of relaxation experiments were performed to investigate the voltage dependency of the increase in gK induced by FMRFamide. The results obtained from these experiments show that the number of channels open at any one time increased with depolarization. The number of channels open at -120 mV was only 60% of the number open at -50 mV. This contrasts with the increase in gK activated by ACh in Aplysia neurones, where the number of channels open at any one time decreased with depolarization (Marty and Ascher, 1978).

The simplest model proposed for transmitter operated channels is that of a conformational change between a closed (TR) and an open (TR^*) state of the receptor-channel complex following the binding of transmitter:

$$T + R \longleftrightarrow TR \underset{q}{\overset{p}{\rightleftharpoons}} TR^{*} \qquad (4.1)$$

During bath application of transmitter, this reaction is presumed to be at equilibrium (Magelby and Stevens, 1972; Dionne and Stevens, 1975; Neher and Sakmann, 1975). Following a voltage perturbation, there is an instantaneous jump, after which, if p and/or q are voltage dependent, the current relaxes to its new equilibrium value with a time constant (τ) described by:

$$\tau = 1/(p + q) \qquad (4.2)$$

During the application of low concentrations of transmitter, the fraction of open channels, $p/(p + q)$, will be small (Ascher, Marty and Nield, 1978), i.e. $p << q$, and τ simplifies to $1/q$. By definition, $1/q$ is the mean lifetime of the open channels. From the relaxation time constants of the FMRFamide induced conductance at -50 mV, a value of around 130 ms was obtained for the mean lifetime of the open channels, assuming that the fraction of channels opened by FMRFamide was small (i.e. $p << q$). An error in the relaxation currents may arise from poorly clamped axonal processes, as axotomy of the neurones was not performed (Finkel, 1983). This may explain the variation observed between the time constants of the current relaxations obtained from different neurones. Furthermore, the assumption that

$p \ll q$ may not necessarily hold under the conditions of these experiments. If the value of p was not significantly smaller than q, then the value quoted for the mean open lifetime would be too small. A decrease in the time constants of current relaxations with increasing dose of ACh has been reported in Aplysia neurones by Marchais and Marty (1979).

The voltage dependency of the current induced by ionophoretically applied FMRFamide may not necessarily reflect the voltage dependency of the current induced by synaptically released FMRFamide. The main difference between these two situations would be the length of time that FMRFamide would be present to activate the receptors. It must be noted that the following argument is purely hypothetical, as no evidence for postsynaptic currents resulting from nerve released FMRFamide exists to date. If it is assumed that nerve released FMRFamide is present for only a very short time compared with the rate of closing of the channels (q), then the amplitude of the current would be a function involving only the opening rate of the channels (p), whereas the time constant of the current decay would equal the mean open lifetime of the channels (1/q). Therefore, any voltage dependency of the amplitude of the synaptic current would reflect the voltage dependency of p, while the voltage dependency of the decay of the current would reflect the voltage dependency of q. However, FMRFamide applied by

ionophoresis may linger for a longer time than nerve released FMRFamide, allowing reaction (4.1) to reach equilibrium. Therefore, the amplitude of ionophoretically applied FMRFamide would depend on both p and q, the voltage dependency of both these parameters would thus be reflected in the amplitude of the ionophoretically induced current. A similar argument was applied by Dionne and Stevens (1975) to describe the difference in the voltage dependency between nerve evoked end plate currents, and ionophoretically evoked end plate currents in the frog neuromuscular junction. During a long burst of presynaptic action potentials, the sustained release of FMRFamide which might occur may, however, result in a postsynaptic current with a voltage dependency resembling that of an ionophoretic pulse of FMRFamide.

4.3 THE DEPOLARIZING RESPONSE INDUCED BY FMRFamide.

4.3.1 THE IONIC MECHANISM OF THE DEPOLARIZATION.

The reversal potential of the hyperpolarization (ca -80 mV) was easily observed, and so aided the determination of the ionic species carrying the charge during the response. A reversal of the current producing the depolarizing response was not obtained. The reason for this was that the steady state currents required to voltage clamp the neurones at positive membrane potentials were outwith the capabilities of the voltage clamp apparatus used. However, ion substitution experiments and the use of channel blockers provided reasonable evidence to support Na^+ ions as the main charge carriers.

The removal of Na from the external solution completely abolished the response, which indicated that a large component of the current flowing during the response was carried by Na^+ ions. In experiments where external Na was reduced, NaCl was replaced by sucrose, as many other compounds used to substitute for Na have channel blocking actions, for example Tris (Ascher, Marty and Nield, 1978) and glucosamine (Marchais and Marty, 1980) decrease the total open time of ACh induced channels in Aplysia; Tris also blocks ACh induced currents in Helix (Witte, Speckmann, and Walden, 1985).

Marchais and Marty (1979) reported that external Na^+ ions (and some other permeant ions) bind to the open ACh channel, thereby reducing the closing rate of these channels. Thus reducing the external Na concentration increased the rate of closing of the ACh activated channels. If Na^+ ions have a similar role in the FMRFamide response, then reducing the external Na concentration may decrease the amplitude of the FMRFamide current more than that expected from the reduced driving force alone.

Extrapolation of the FMRFamide response observed under voltage clamp conditions gave a reversal potential significantly more positive than zero. The ACh induced depolarizations in these neurones consists of an increase in gK, gNa and gCa, which leads to a reversal potential close to zero (Witte, Speckmann and Walden, 1985). The reversal potential for the 5-HT induced depolarizations in Helix is also approximately zero (Gerschenfeld and Paupardin-Tritsch, 1974).

4.3.2 THE EFFECT OF K CHANNEL BLOCKERS ON THE INCREASE IN gNa.

The K channel blockers TEA and intracellular Cs, which markedly attenuated the increase in gK, were without effect on this response. The lack of effect of Cs on the response proved useful, as Cs could be injected into the cell to reduce the steady state outward currents, thereby increasing the range of potentials over which the cell could be voltage clamped. With intracellular Cs present, the FMRFamide current was still inward at +20 mV. The relationship between holding potential and response amplitude in the presence of Cs appeared linear on first inspection. Extrapolation of a straight line fitted to the data gave a reversal potential for the response of +40 mV. However, application of the GHK equation to the data, assuming a pure increase in permeability to Na, gave a better fit than a straight line, and using an internal Na concentration of 3.8 mM (cf. Meech and Thomas, 1977) yielded a reversal potential of +80 mV.

The reversal potential of responses recorded under voltage clamp conditions may not necessarily indicate the reversal of the single channel currents associated with the response. This is especially true of depolarizing responses, as some of the ions which may enter through the agonist induced channels could themselves activate a

secondary conductance, usually to K^+ ions. A rise in intracellular Ca is well documented to cause an increase in membrane permeability to K^+ ions. Recently, an influx of Na^+ ions has been reported to activate a K current in cultured avian neurones (Bader, Bernhein and Bertrand, 1985). This current appears to have little voltage dependent inactivation, and may be induced following Na entry during action potentials and possibly during Na mediated post synaptic potentials. A Na sensitive K current has also been described in crayfish neurones (Hartung, 1985).

The relationship between response amplitude and membrane potential in the presence of intracellular Cs may therefore be more positive than the reversal potential in normal situations. Ascher, Marty and Nield (1978) have shown that during the ACh induced current in voltage clamped Aplysia neurones, that sufficient Ca^{2+} ions enter through the ACh channel to activate a Ca dependent K current. If the FMRFamide activated channels were permeable to Ca, then during the presence of Cs the entry of Ca would be unable to activate a K current, thus under these conditions the reversal potential of the FMRFamide induced current would be more positive than during control conditions.

4.4 THE VOLTAGE DEPENDENT RESPONSE INDUCED BY FMRFamide.

4.4.1 THE IONIC MECHANISM OF THE VOLTAGE DEPENDENT RESPONSE.

Another response produced by FMRFamide was a voltage dependent inward current. This response, which occured in the C1 neurone, proved difficult to observe using FMRFamide as the agonist because the large increase in gK produced by FMRFamide in this neurone masked the voltage dependent response. The FMRFamide analogue, YGGFMRFamide, was more potent at inducing the voltage dependent effect (Cottrell, 1982a), and was therefore the agonist used to activate this response.

The voltage dependent response was absent at resting membrane potentials, and was only detected when the neurone was artificially depolarized. Under voltage clamp conditions, the response was observed as an inward current when the holding potential was more positive than -40 mV. Similar voltage dependent inward currents have been observed with 5-HT in this neurone (Cottrell, 1981; Barnes Cottrell and Dunbar, 1986) and in Aplysia neurones (Klein and Kandel, 1980; Pellmar and Carpenter, 1980). In bullfrog sympathetic ganglia muscarinic agonists, LHRH and substance P decrease a K current (the M-current) which leads to an inward current which is voltage sensitive

(Brown and Adams, 1980; Adams and Brown, 1980; Adams, Brown and Jones, 1983).

The YGGFMRFamide induced inward current in the C1 neurone was always observed under conditions where the steady state current of the neurone was outward. During voltage clamp, it is the net current flowing across the neuronal membrane that is measured. This current is mainly carried by K^+ ions at holding potentials positive to -40 mV. The voltage dependent inward current induced by YGGFMRFamide could therefore be the result of a decrease in one or more of the components of this outward current.

If this response was a decrease in a K current, then the overall membrane conductance should decrease during the response. An indication of the change in membrane conductance occurring during the response was obtained when hyperpolarizing command pulses of constant potential were applied at regular intervals to the neurone. The amplitude of the resulting current deflections which was monitored during the response, was decreased; however, this could be interpreted as either an increase or decrease in membrane conductance. Consider a C1 neurone voltage clamped at -20 mV, with 20 mV hyperpolarizing command potentials applied to the cell at regular intervals. Because of the strong voltage dependent nature of the response, there would be an inward current at

-20 mV, but no current at -40 mV. Therefore, whether the response was due to a voltage dependent inward current such as a Ca current, or to a voltage dependent decrease in an outward current, such as a K current, the amplitude of the resulting current deflections would decrease by a similar amount during the response. A similar argument was given by Dingledine (1983) during his investigation of a voltage dependent Ca current activated by NMDA in rat hippocampal neurones.

Cottrell (1979, 1982a) showed that the response was unaltered by replacing NaCl with Tris/HCl or sucrose, suggesting that neither Na^+ nor Cl^- ions were involved. Furthermore, when Ba was substituted for Ca, the response was abolished. These results imply that the response is due to the reduction of an outward K current, and not to a voltage dependent increase in gNa and/or gCa. In the same neurone (the C1 neurone) 5-HT induces a voltage dependent response with almost identical properties (Cottrell, 1981). Both responses are sensitive to extracellular K concentration and are blocked by low concentrations of Co (Cottrell, 1982b; Barnes, Cottrell and Dunbar, 1985). A role of Ca in the production of the response was suggested by the inhibitory action of Co on the response. Co is a well known inhibitor of Ca currents, both in these neurones (Akaike, Lee and Brown, 1978; Barnes, Cottrell and Dunbar, 1985) and in other excitable tissues (Hagiwara and Byerly, 1981). This effect of Co could be explained

if YGGFMRFamide (and 5-HT) suppressed a Ca activated K current in this neurone. Both histamine and noradrenaline have been shown to reduce a Ca activated K current in hippocampal pyramidal cells (Haas and Konnerth, 1983). If this were the case here, then artificially increasing the amount of Ca activated K current should increase the size of the response. One way of increasing the Ca activated K current is to inject Ca into the cell (Meech, 1974; Meech and Standen, 1975; Hermann and Gorman, 1981a,b; Hofmeier and Lux, 1981).

4.4.2 THE EFFECT OF INTRACELLULAR Ca INJECTION.

Intracellular injection of Ca into the C1 neurone during voltage clamp produced an outward current, and a concomitant increase in membrane conductance. This outward current was reversed when the holding potential was more negative than E_K. Furthermore, Cottrell (1982a) showed that the reversal potential of this Ca induced current was dependent on the external K concentration. It was therefore concluded that the outward current evoked by Ca injection was carried by K^+ ions.

Cottrell (1982a) showed that the amplitude of the peptide induced response was increased following the injection of Ca into the neurone. This experiment was repeated here and the results of Cottrell (1982a) were confirmed. The amplitude of the response appeared to

increase linearly with the amount of outward current induced following the injection of Ca into the neurone. This implied that YGGFMRFamide suppressed the outward K current induced by Ca injection. However, this experiment was technically difficult as it relied on the injection of Ca producing a large, well sustained outward current, which presumably depended on a number of factors controlling the level of free Ca^{2+} ions close to the inner surface of the membrane (Barish and Thompson, 1983). Well sustained outward currents were rarely the case. Following the injection of Ca into the cell from an ionophoretic pipette, Rose and Loewenstein (1975) have shown that the diffusion of free Ca^{2+} ions is greatly restricted by intracellular Ca sequestration mechanisms. If this were the case in these experiments, then the activation of the outward K current by the injected Ca may only occur over a limited area of the neuronal membrane, depending on the precise position of the tip of the Ca electrode. Thus, if the local application of YGGFMRFamide only reached an area of membrane where there was none, or very little K current activated following Ca injection, then the peptide induced response may not be very different to that produced before the injection of Ca, even though a significant increase in outward current occurred.

4.4.3 THE INVOLVEMENT OF A SECOND MESSENGER.

Many of the currents induced by Ca injection waned rather rapidly. This was attributed to the sequestration of free Ca by cellular organelles and proteins. The experiments in which CCmP was applied to the external solution were designed to overcome this sequestration by causing a release of stored Ca (Meech and Thomas, 1980). Although this compound was successful in prolonging the outward current activated by Ca, it completely abolished the YGGFMRFamide induced current. CCmP is a powerful metabolic inhibitor (Heytler and Prichard, 1962), therefore this result implied that a metabolic process, possibly a second messenger system, is involved in the generation of the voltage dependent response.

Increasing the cyclic nucleotide levels in the C1 neurone produced effects very similar to the peptide induced response. Application of theophylline reduced the outward current observed in the C1 neurone under voltage clamp. During the action of theophylline, application of peptide failed to produce a further reduction in outward current. The reduction in outward current produced by theophylline suggests that there is a basal production of cyclic nucleotides in the C1 neurone, and that these may regulate the K permeability of the membrane to some extent.

There have been many reports of molluscan voltage dependent responses mediated by cAMP. A 5-HT evoked voltage dependent decrease in gK in identified neurones of the suboesophageal ganglia of Helix is mimicked by intracellular cAMP injection, and abolished by 100 uM IBMX (Deterre, Paupardin-Tritsch, Bockaert and Gerschenfeld, 1981). Like theophylline, the phosphodiesterase inhibitor IBMX reduced the outward steady state currents in these neurones. However, a lower concentration of IBMX augmented the 5-HT response (Deterre et al., 1981). Both 5-HT and dopamine have been shown to stimulate adenylate cyclase in Helix neurones (Deterre, Paupardin-Tritsch, Bockaert and Gerschenfeld, 1982). In Aplysia RB and LB neurones, 5-HT induces a voltage dependent inward current that is likely to be carried by Ca^{2+} ions (Pellmar and Carpenter, 1980; Pellmar, 1984). Although both 5-HT and cAMP induce similar, voltage dependent, inward currents in these Aplysia neurones, Pellmar (1981) concluded that it was unlikely that this response was mediated by cAMP on the basis that low concentrations of phosphodiesterase inhibitors failed to enhance the response to 5-HT. This argument should be considered here as only a high concentration (1 mM) of theophylline was tested, and it should not be assumed that a low concentration of theophylline would enhance the response, as expected of a response mediated by cAMP. The voltage dependent response to 5-HT in Aplysia sensory neurones is different to that

in the LB and RB neurones mentioned above, being the result of a decrease in gK which involves a second messenger, probably cAMP (Klein and Kandel, 1980; Klein, Camardo and Kandel, 1982). Recently, a FMRFamide induced reduction of a cAMP sensitive K current has been described in neurones of the suboesophageal ganglia of Helix (Colombaioni, Paupardin-Tritsch, Vidal and Gerschenfeld, 1985). Both FMRFamide and 5-HT have been shown to activate adenylate cyclase activity in Mercenaria ventricles (Higgins, Price and Greenberg, 1978).

4.4.4 ADDITIONAL EVIDENCE FOR A SECOND MESSENGER ROLE FROM SINGLE CHANNEL STUDIES.

Experiments performed on the C1 neurone of Helix showed that the activity of K channels recorded from a cell attached patch was reduced when the FMRFamide analogue FnLRFamide was applied to the cell surface outside the patch (Cottrell, Davies and Green, 1984). In the cell attached mode, a very high resistance seal (ca. $10^{12}\Omega$) is formed between the inside of the patch electrode and the extracellular solution by the adherence of the membrane to the tip of the electrode. During the application of substances to the extracellular fluid surrounding the neurone, the substances do not have access to the inside of the patch pipette. Therefore, the action of FnLRFamide on K channels under the patch pipette must occur via a second messenger system following activation

of the receptors.

The K channels modulated by FnLRFamide appear to be the same as those modulated by 5-HT (Cottrell, Davies and Green, 1984; Barnes, 1984; Barnes, Cottrell and Dunbar, 1986). The channels, as recorded from an inside out patch, are not sensitive to changes in Ca concentrations on the inside surface (Barnes, Cottrell and Dunbar, 1985). Thus it appears unlikely that the peptides suppress a Ca activated K current, unless there was another type of Ca sensitive K channel which was suppressed by the peptides, but which remained undetected during the experiments.

Application of these methods to Aplysia sensory neurones by Siegelbaum, Camardo and Kandel (1982), revealed that the activity of K channels recorded from a cell attached patch were reduced both by application of 5-HT and by intracellular injection of cAMP. Subsequent experiments using an inside out patch have shown that these channels are sensitive to phosphorylated protein kinase C (Shuster, Camardo, Siegelbaum and Kandel, 1985), but insensitive to Ca^{2+} ions.

Further evidence contradicting a FMRFamide suppression of a Ca activated K current was the lack of effect of the organic Ca channel blocker verapamil on either the response to YGGFMRFamide and 5-HT or on the I-V

curve of the C1 neurone between -70 and -15 mV. Verapamil has been shown to block the Ca current in Helix neurones (Akaike, Lee and Brown, 1978; Barnes, Cottrell and Dunbar, 1985). Therefore, it appears unlikely that there is a significant K current activated by an influx of Ca^{2+} ions across the membrane of the C1 neurone over the potential range indicated. However, the possibility that internal release of Ca leading to the activation of a K current cannot be disregarded (Akaike, Brown, Dahl, Higashi Isenberg, Tsuda and Yatani, 1983). The reduction of outward current and the blockade of the response by Co seems to relate to an action of Co which is additional to its well known action as a Ca channel blocker (see also Barnes, Cottrell and Dunbar, 1985).

4.5 FLRFamide AND FIRFamide PRODUCE RESPONSES SIMILAR TO FMRFamide.

Application of FLRFamide and FIRFamide onto neurones which responded to FMRFamide invariably produced similar effects to those produced by FMRFamide. The ionic mechanisms were the same as those induced by FMRFamide. The hyperpolarizing response induced by FLRFamide followed E_K within a few millivolts after increasing the external K concentration from 5 to 15 mM, and the depolarizing response to FLRFamide was attenuated by lowering the external Na concentration. However, the effects of channel blockers such as TEA and intracellular Cs were not investigated.

Apart from the ionic mechanisms, other properties of the FMRFamide responses were shared with those induced by FLRFamide and FIRFamide. The time course and voltage dependency of the increase in gK produced by FLRFamide resembled closely the FMRFamide induced increase in gK. In addition, the maximum response and potency of both peptides were similar. The increase in gNa produced by all three peptides was also similar, the desensitization normally observed with FMRFamide was evident with FLRFamide and FIRFamide. The above similarities between the responses induced by FMRFamide and its analogues where Met^2 was substituted for Leu or Ile imply that these

peptides act on the same receptors. Substitution of the Met^2 residue of FMRFamide does not significantly alter the biological activity, or the potency, when tested on many molluscan muscles (Painter, Morley and Price, 1982).

Further evidence suggesting that these tetrapeptides act on the same receptors was the cross-desensitization observed between them in activating the increase in gNa. However, the possibility that the peptides interact to produce a common desensitizing step, leading to an increase in gNa is unlikely, but cannot be disregarded. A similar scheme has been proposed by Ascher and Chesnoy-Marchais (1982) to explain the cross-desensitization observed between carbachol, dopamine and histamine in Aplysia neurones.

4.6 MULTIPLE RESPONSES MAY OCCUR ON SINGLE NEURONES.

Combinations of the different ionic actions of FMRFamide occurred in some of the neurones investigated, notable examples being the C1 and F2 neurones. The C1 neurone responded to FMRFamide with a simultaneous increase and decrease in gK (Cottrell, 1982a). The response of various C1 neurones to FMRFamide was often only an outward current, which declined in amplitude as the neurone was held at more depolarized potentials. The FMRFamide analogue YGGFMRFamide was much more potent at inducing the voltage dependent component of the response

in the C1 neurone. Because both of these responses involved K^+ ions, it was not possible to separate them satisfactorily, either pharmacologically or by ion substitution methods. Although some K channel blockers inhibited one response more than the other, for example TEA which was more potent in blocking the increase in gK (Cottrell, 1982a), an action on the other K response was always likely. Separation by voltage was possible due to the strong voltage dependency of the decrease in gK, which was absent at potentials more negative than -40 mV. Under these conditions, the increase in gK observed in the C1 neurone was identical to that observed in other neurones which responded with an apparently pure increase in gK at all potentials investigated.

The response of the F2 neurone to FMRFamide involved an increase in both gNa and gK. The two components of the F2 response could be separated by pharmacological and ion substitution methods. Zero Na solution abolished the Na component completely, revealing a pure K component, while intracellular Cs blocked the K component, leaving the Na component unaltered.

An increase in both gNa and gK can occur by four different mechanisms: (1) a single receptor associated with a single channel which is permeable to both Na and K, (2) a single receptor controlling the gating of two channels, one channel selective for Na, the other for K,

(3) two independent populations of channels, some being selective for Na and the others for K, with the gating of each channel controlled by a single receptor which is of a common type, (4) two populations of channels and two populations of receptors, one type of receptor associated with Na selective channels, the other type with K selective channels (Cottrell, Davies and Green, 1984).

There was a large variation in the reversal potential of the FMRFamide responses recorded from F2 neurones of different preparations. This variation (-55 to -20 mV) was much greater than could be expected from variations in the intracellular concentrations of Na and K in different F2 neurones. It was therefore very unlikely that possibilities (1) and (2) could be candidates for the mechanism of the F2 response, as in both of these cases, the reversal potential of the response should be constant in all F2 neurones. Furthermore, the action of the K channel blockers TEA and Cs in reducing the K component of the response disregards possibility (1), because TEA and Cs would not be able to discriminate between K^+ and Na^+ ions flowing through such a non-selective channel. When the membrane potential was held close to the reversal potential of the response, the currents recorded were biphasic. This is again inconsistent with possibility (1), and although a biphasic response could occur if a receptor/two-channel complex yielded different kinetic constants for the K and Na channel, the constants would

have to be very different to account for the different time courses occurring here.

The subdivisions of the response of the F2 neurone to FMRFamide yielded a K current and a Na current, both of which had identical properties to the individual K and Na currents observed in different neurones. Thus it is concluded that the response of the F2 neurone to FMRFamide arises as a result of the increase in gK and the increase in gNa responses occurring in combination on this neurone.

The characteristics of the response of the F2 neurone to FMRFamide; variable reversal potential, biphasic response and separation of the current components by channel blockers, implies that the response is the result of either the activation of two populations of channels by a single receptor type (case 3),or the activation of two populations of channels, each type of channel associated with different receptors (case 4). The variability of the reversal potential may be explained by a topographical distribution of the channel types, such as that described for ACh receptors on a Navanax neurone (Levitan and Tauc, 1972). The ratio of Na to K channels activated would depend on their respective distribution close to the site of FMRFamide application. A variation in the reversal potential can also be explained if the relative potency of FMRFamide towards activating the two types of channels was

different (see later).

4.7 ARE THE RECEPTORS MEDIATING THE INCREASE IN gK AND THE INCREASE IN gNa DIFFERENT?

4.7.1 DIFFERENCES IN DESENSITIZATION PROPERTIES.

Apart from the ionic mechanisms, the most striking difference between the FMRFamide induced increase in gK and gNa is the rapid desensitization associated with the increase in gNa. Desensitization of the increase in gK was not evident. This is consistent with the existence of two types of receptors, one type mediating the increase in gNa, which can become desensitized, the other type mediating the increase in gK, which does not enter a desensitized state. Although Katz and Theslef (1957) proposed that the desensitization of the ACh response of the neuromuscular junction was at the receptor level, this may not always be the case. Desensitization of a response may occur at a step other than the receptor, such as the production of a second messenger (Sibley and Lefkowitz, 1985). This situation cannot be ruled out in the desensitization of the increase in gNa induced by FMRFamide. Therefore it is not possible to use this difference between the increase in gK and the increase in gNa as conclusive evidence for the existence of different receptors mediating the two responses.

A multireceptor system, i.e. case (4) above, occurs for all transmitter substances studied in molluscan ganglia (Ascher and Kehoe, 1975). The transmitter substances most studied have been ACh (Kehoe, 1972a,b), 5-HT (Gerschenfeld and Paupardin-Tritsch, 1974) and dopamine (Ascher, 1972; Berry and Cottrell, 1979). The use of specific antagonists greatly facilitated the classification of receptor types for these transmitters (Ascher and Kehoe, 1975). Unfortunately, no antagonist compounds for FMRFamide receptor(s) have been discovered to date, making the distinction between the occurrence of a single type of FMRFamide receptor or multiple FMRFamide receptors difficult. An attempt was made to distinguish between the two situations by comparing the potency of FMRFamide at producing the different types of responses and also by comparing the actions of FMRFamide analogues with those of FMRFamide on identified neurones.

4.7.2 DOSE-RESPONSE CURVES OF FMRFamide.

The concentration of FMRFamide required to activate the increase in gNa was approximately 100 times greater than that required for the activation of the increase in gK. This was the ratio obtained by comparing the threshold concentrations for the activation of the two responses. A value for the concentration of FMRFamide required to produce a half maximal increase in gNa could

not be obtained because the prominent desensitization of this response made it impossible to observe a true maximum response. The possibility that the high threshold value for the increase in gNa was an artefact of desensitization was eliminated by checking the desensitization of ionophoretically applied FMRFamide after application of the peptide to the bath. At a bath concentration slightly lower than threshold, there was no apparent desensitization of ionophoretically applied FMRFamide. The dose response relationship for the activation of the increase in gK was performed at a holding potential of -45 mV, while that of the increase in gNa was performed at a holding potential of -70 mV. As a result, an exaggeration of the difference in threshold values between the two responses resulting from a difference in holding potential cannot be ruled out as voltage dependent dissociation constants have been described previously (Onsager, 1934).

4.7.3 A MODEL FOR THE RESPONSE OF THE F2 NEURONE BASED ON THE DIFFERENT POTENCY OF FMRFamide TOWARDS THE INCREASE IN gK AND THE INCREASE IN gNa.

The large difference in the potency of FMRFamide in activating these two different responses can explain the variability of the reversal potential of the response of F2 neurones to FMRFamide. If a low concentration of FMRFamide reaches the F2 neurone, then the K component

will predominate, however, if a high concentration reaches the neurone, a substantial Na component will be present, depending of course on the membrane potential. Many factors can account for a different concentration of peptide reaching the F2 neurone, such as the transport number of FMRFamide, the duration and magnitude of the ejection current and the position of the ionophoresis electrode relative to the receptors. If, when using a particular ionophoretic pipette, the transport number is assumed constant and the ejection current and duration were the same, the predominant factor affecting the concentration of FMRFamide reaching the receptors is the relative position of the tip of the pipette to the receptors. A variation of this parameter is very likely to occur between different preparations, and a simple model, based on the diffusion equations, was developed which could explain how the variation in reversal potential might arise as a result of a variation of the position of the peptide electrode relative to the receptors.

The model was based on a neurone subjected to locally applied agonist during voltage clamp. The agonist induces an increase in gK and an increase in gNa by interacting with two types of receptors. Both holding potential and the distance of the agonist pipette could be varied during the execution of the programme, which was written in basic to run on a BBC microcomputer (with a 6502 processor).

Membrane currents were described by:

$$I = V.(gT) - (gK.E_K + gNa.E_{Na} + gCl.E_{Cl}) \qquad (4.3)$$

where

$$gT = gK + gNa + gCl$$

and V is the holding potential (Ginsborg, 1973). The agonist induced increase in gNa and gK were described by:

$$gNa = gNa_{max}.C/(C + Kd_{Na}) \qquad (4.4a)$$

and

$$gK = gK_{max}.C/(C + Kd_K) \qquad (4.4b)$$

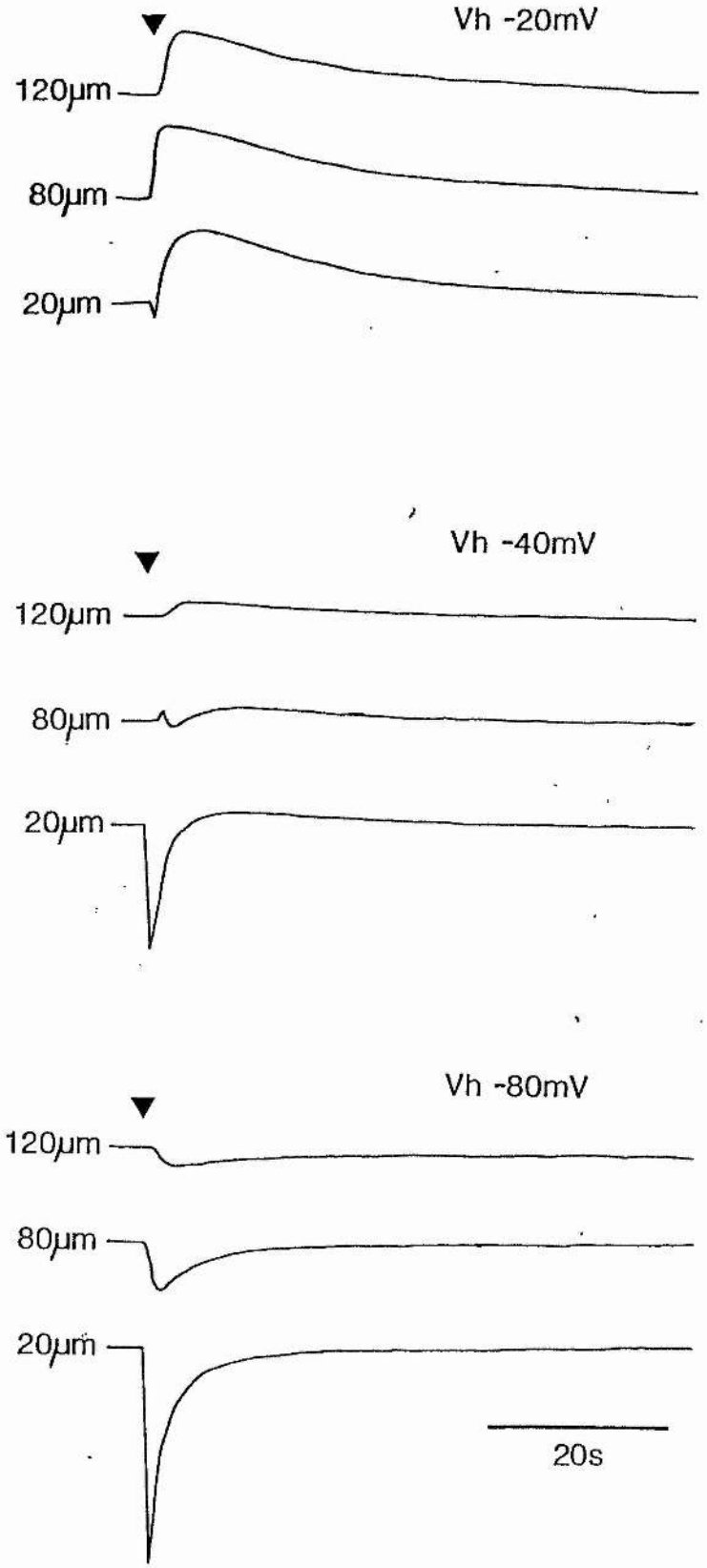
Values of $1.8x10^{-6}$ and $50x10^{-6}$ M were given to Kd_K and Kd_{Na} espectively.

The diffusion from the pipette was calculated assuming an instantaneous release of agonist, and that the surface of the neurone was represented by a small point. The concentration (C) of agonist at the surface of the neurone at time t was given by:

$$C = M.(4\pi Dt)^{-1.5}\exp[-(x^2/4Dt)] \qquad (4.5)$$

where M is the amount of agonist released from the pipette, D is the diffusion coefficient of the agonist, and x is the distance of the agonist pipette from the cell. The effect of placing the pipette at various

Fig.4:1. The effect of the distance of an ionophoresis pipette from a population of two types of receptors, one type with a dissociation constant of $1.8x10^{-6}$ M mediating an increase in gK, the other type with a dissociation constant of $50x10^{-6}$ M mediating an increase in gNa. The neurone is voltage clamped at three different holding potentials (Vh). Increasing the distance of the pipette results in a reduction of the relative component of the increase in gNa in the response. The amount (M) of substance ejected was set at $1x10^{-15}$ mol, and the coefficient of diffusion was set at $5x10^{-9}$ m^2/s.


Vh -20mV
120μm
80μm
20μm
Vh -40mV
120μm
80μm
20μm
Vh -80mV
120μm
80μm
20μm
20s

distances from the neurone is shown in Fig.4:1.

This simple model demonstrates how the reversal potential of a multireceptor response can vary with the concentration of agonist reaching the receptors if the value of the dissociation constants for the receptor types are different. The variability of the reversal potential of the F2 response to FMRFamide can be explained in part by this model, however, regional differences between the distribution of the two types of receptor-channel complexes on the F2 neurone may well occur.

4.8 FMRFamide PEPTIDES WITH N-TERMINAL EXTENSIONS.

4.8.1 ACTIONS OF THE N-TERMINAL EXTENDED ANALOGUES.

Substitution of Met^2 of FMRFamide for Leu or Ile had little effect on either biological activity or potency, the effects of the resulting analogues were, on the whole, indistinguishable from those of FMRFamide. Extension of the N-terminal by Tyr-Gly-Gly- produces YGGFMRFamide, which is reported to be a potent analogue of FMRFamide, both peptides being equipotent and having identical actions on many molluscan muscles (Greenberg, Painter and Price, 1981). Differential actions have, however, been observed in other molluscan muscles, such as the tentacle retractor muscle of Helix (Cottrell, Greenberg and Price, 1983), and Mytilus ABRM (Muneoka and Matsuura, 1985).

Application of YGGFMRFamide onto Helix neurones produced actions which were different to those induced by FMRFamide. Several other N-terminally extended peptides which were tested on Helix neurones gave responses resembling those of YGGFMRFamide (Cottrell and Davies, 1985).

YGGFMRFamide is more potent than FMRFamide at inducing the voltage dependent decrease in gK (Cottrell, 1982a). This was also found to be true for the extended analogue pQDPFLRFamide. Both of these peptides often only produced a weak outward K current in the C1 neurone. In other neurones, however, a comparatively fast hyperpolarization resulted from local application of both YGGFMRFamide and pQDPFLRFamide. Two other N-terminally extended analogues, PFLRFamide and GDPFLRFamide, also evoked this fast hyperpolarization.

Experiments performed under voltage clamp conditions showed that this response was evident as an outward current, which reversed to an inward current at potentials more negative than -80 mV. This response was sensitive to external K concentration, and was attenuated by TEA and intracellular Cs^+ ions. The attenuation by TEA was somewhat less than that of the FMRFamide induced slow increase in gK. It is concluded that this fast hyperpolarization was mediated by K^+ ions. This response is termed the fast increase in gK to distinguish it from

the slow increase in gK induced by the tetrapeptides.

It could be argued that these two K responses are not different, but that the difference in duration arose simply because of a difference in potency between FMRFamide and the extended peptides at producing the same increase in gK. This is unlikely on account of a number of observations: (1), the fast increase in gK was not observed with any of the tetrapeptides, (2), the fast increase in gK was occasionally observed in cells where FMRFamide did not induce the slow increase in gK, (3), the heptapeptides could weakly activate the slow increase in gK, and (4), a combination of the fast and slow increase in gK was sometimes induced by the extended peptides on a single neurone. In one neurone where both fast and slow increases in gK were observed, the peptide (pQDPFLRFamide) pipette was placed in different positions around the soma of the neurone. This resulted in changes in the proportion of fast and slow components in the the overall response. Furthermore, the peak of the fast and slow components were temporally separated, the peak of the slow component always occurring later than that of the fast component. This can not be attributed to a spatial distribution of a single type of receptor, as such a situation should produce a single peaked response (Purves, 1977). From these observations, it seems likely that the two increases in gK are two different responses, and not merely an artefact of concentration and potency. The

results are also consistent with the occurrence of different receptors associated with the fast and slow increase in gK.

4.8.2 DIFFERENCES BETWEEN THE TETRAPEPTIDES AND THE N-TERMINAL EXTENDED ANALOGUES.

Apart from the differences in the increase in gK activated by the tetrapeptides and the N-terminally extended analogues, another major difference between them is the lack of activation of the increase in gNa by the extended peptides. This produced significant differences when comparisons were made of the actions of the extended peptides with those of FMRFamide on the F2 neurone. While FMRFamide produced an inward, depolarizing current, the extended peptides produced an outward, hyperpolarizing current.

Contrasting responses were noticed first between FMRFamide and YGGFMRFamide. It was conceivable that the differences arose because of the existence of distinct receptor types, one type recognising FMRFamide peptides, and the other type being an opioid type receptor, recognising the N-terminal Met-enkephalin sequence of YGGFMRFamide. An opioid receptor system has been reported in Helix pomatia neurones (Stefano, S.-Rozsa and Hiripi, 1980). However, the interaction of YGGFMRFamide with an opioid receptor seemed unlikely here, as neither YGGFM nor

YGGFMRF induced responses in neurones where YGGFMRFamide produced substantial responses. Furthermore, the actions of pQDPFLRFamide and PFLRFamide were very similar to those of YGGFMRFamide. With the only common sequences in these analogues being F-RFamide, this also makes it unlikely that the biological action of YGGFMRFamide, and also pQDPFLRFamide and PFLRFamide resides only in the N-terminal. Two other peptides which gave no responses when compared with pQDPFLRFamide were pQDPFLRLamide and pQDPFLRIamide, the only difference being that Phe^7 was substituted for Leu or Ile. The failure of these peptides to induce responses again strongly suggests that the activity of pQDPFLRFamide does not only reside in the N-terminal part of the molecule, but that the C-terminal sequence is also vital for the biological activity of the extended FMRFamide analogues.

4.9 AN ANALOGY WITH THE OPIOID SYSTEM.

One of the peptide receptor systems which has been thoroughly studied is the opioid system. There are at least four well characterized opioid binding sites, the δ-, μ-, k- and ϵ-opioid receptors. The endogenous opioids interact with all four of these receptors to varying degrees. The affinity of Met-enkephalin towards the δ-receptor is altered towards both δ- and μ-receptors by extending the C-terminal by -Arg-Phe to give YGGFMRF (Kosterlitz, 1985). Decreasing

β-endorphin(1-31) to β-endorphin(1-17) by sequentially removing C-terminal amino acids, does not alter the affinity of the resulting analogues towards either δ- or μ- sites, however, the affinity towards the ε - site decreases markedly once β-endorphin(1-22) is reached (Schultz, Wuster and Herz, 1981). These are just two examples where changing the length of the peptide chain alters the interaction of the resulting compounds with the different receptors.

An analogy exists between the opioid system and the FMRFamide system, in that extending the N-terminal of FMRFamide also alters the biological activity, possibly by directing the resulting analogue towards a different receptor. This theory was proposed for the opioid peptide dynorphin. Decreasing dynorphin(1-13) to dynorphin(1-5) (Leu-enkephalin) resulted in major decreases in the potency of this peptide, especially with removal of Lys^{13}, Lys^{11} and Arg^{7} (Chavkin and Goldstein. 1981; James, Fischli and Goldstein, 1984). The amino acids 1-4 (YGGF) were regarded as the opioid message sequence, while 5-13 was the address sequence, which enhanced the potency towards the κ-receptor. If it is assumed that the four responses observed with FMRFamide peptides are produced by the activation of four distinct receptors, then N-terminal extensions may be regarded as address sequences, enhancing the potency towards the receptors associated with the fast increase in gK and the decrease in gK. It is not known

whether increasing the FMRFamide chain by one amino acid is enough to alter the potency, or whether the amino acid has to be proline. However, there appears to be little similarity between the YGG- extension and the other extension sequences.

4.10 A HYPOTHESIS EXPLAINING THE MULTIPLE RESPONSES INDUCED BY FMRFamide PEPTIDES.

The four responses induced by FMRFamide peptides differ not only in their ionic mechanisms, but also in their relative sensitivity to the various FMRFamide analogues tested. The results obtained are consistent with the presence of four FMRFamide-peptide receptors on Helix neurones, and are tentatively listed below:

RECEPTOR	RESPONSE
FaR1	increase in gNa
FaR2	slow increase in gK
FaR3	fast increase in gK
FaR4	decrease in gK

FaR1 and FaR2 are activated mainly by the tetrapeptides, while the N-terminally extended peptides activate mainly FaR3 and FaR4. Conclusive proof of this proposal, however, awaits the discovery of selective antagonists for these putative receptors.

4.11 PHYSIOLOGICAL ROLE OF FMRFamide PEPTIDES.

The ionic actions of the responses observed suggest that FMRFamide peptides, if released in sufficient quantities from nerve terminals, could act as interneuronal messengers. The increase in gNa could have an excitatory role, while both the fast and slow increase in gK would lead to inhibition. These responses, which involve an increase in conductance, are similar to many well established responses to synaptically released transmitter substances (Kehoe, 1972a; Gerschenfeld and Paupardin-Tritsch, 1974; Berry and Cottrell, 1979). The reason for the combination of the increase in gNa and the increase in gK on the F2 neurone is not known. However, the difference in sensitivity of these two responses to FMRFamide, together with the rapid desensitization of the increase in gNa, suggests that the response of the F2 neurone may be a function of the amount of FMRFamide released and the duration of release.

The voltage dependent decrease in gK may have a more subtle, but nonetheless influential, action on the electrical properties of individual neurones. Due to the decrease in conductance accompanying this response, both the time and space constant of the neurone will increase, thus increasing the efficacy of post-synaptic potentials. A voltage dependent suppression of a K current occurs in

sympathetic ganglion cells following application of LHRH, substance P and muscarinic agonists (Adams and Brown, 1980; Brown and Adams, 1980; Adams Brown and Jones, 1983). This is believed to be the mechanism underlying the slow EPSP and the late slow EPSP in these neurones. The late slow EPSP is probably generated following the release of an LHRH-like peptide (Jan, Jan and Kuffler, 1979; Jan and Jan, 1982). This decrease in a K current leads to an increased excitability of bullfrog ganglion neurones by facilitating incoming excitatory postsynaptic events (Brown, 1983).

The voltage dependent nature of the response to FMRFamide peptides implies a possible modulatory role on action potential duration, and consequently, if occurring in presynaptic terminals, on the modulation of transmitter release (Katz and Miledi, 1967; Llinas, Sugimori and Simon, 1982). In an identified neurone (the A neurone) in the buccal ganglion of Helix, 5-HT produces a voltage dependent response, similar to that induced by FMRFamide peptides in the C1 neurone. Cottrell (1982b) has shown that synaptically released 5-HT also evokes this response in the A neurone, during which there is a concomitant increase in the duration of the action potential and also a decrease in the amount of accommodation during periods of high frequency of action potential firing. A similar response induced by 5-HT in Aplysia sensory neurones is believed to be important in behavioural sensitization

(Klein and Kandel, 1978; Klein, Camardo and Kandel, 1982).

FMRFamide peptides have been located immunohistochemically in Helix ganglia using antibodies directed against the sequence -Met-Arg-Phe-NH_2. In particular, immunoreactivity has been detected in an identified neurone in the cerebral ganglion, termed the C3 neurone (Cottrell, Schot and Dockray, 1983). This neurone projects to the tentacle retractor muscle where FMRFamide reactive neuronal processes are present. Stimulation of the C3 neurone causes a contraction of the tentacle retractor muscle, however, it is not determined whether the contraction arises as a direct result of the release of peptide.

Recently both FMRFamide and pQDPFLRFamide have been extracted from Helix ganglia, in addition to at least one other FMRFamide related peptide (Price, Cottrell, Doble, Greenberg, Jorenby, Lehman & Riehm, 1985). The contrasting responses observed with these two peptides suggest that they may have quite different physiological roles. The release of FMRFamide onto a neurone may have an effect which is quite the opposite to that of the release of pQDPFLRFamide. It is interesting to note that removal of three amino acids from the N-terminal of pQDPFLRFamide yields FLRFamide, which has very different biological properties. If differential processing of the

precursor molecule occurred, then it is conceivable that a certain neurone may release either FLRFamide or pQDPFLRFamide. Such a situation could therefore change particular synapses from being excitatory to inhibitory by altering the processing of the precursor molecule.

The existence of FMRFamide peptides in the nervous system of Helix, together with immunohistochemical localization of these peptides in identified neurones strongly suggest that they may have a physiological role in this mollusc. The potent and diverse effects of FMRFamide peptides on molluscan muscles and neurones enhance this view, and imply a possible neurotransmitter action for these peptides. Furthermore, a continuation of this research on the Helix nervous system should give a valuable insight into neuropeptide physiology.

REFERENCES

ABE, H., INONE, M., MATSUO, T. & OGATA, N. (1983). The effects of vasopressin on electrical activity in the guinea-pig supraoptic nucleus in vitro. J. Physiol. 337:665-685.

ADAMS, J.D., SMITH, J.S. & THOMPSON, S.H. (1980). Ionic currents in molluscan soma. Ann. Rev. Neurosci. 3:141-167.

ADAMS, M.E. & O'SHEA, M. (1983). Peptide cotransmitter at a neuromuscular junction. Science 221:286-289.

ADAMS, P.R. & BROWN, D.A. (1980). Luteinizing hormone-releasing factor and muscarinic agonists act on the same voltage-sensitive K^+-current in bullfrog sympathetic neurones. Br. J. Pharmac. 68:353-355.

ADAMS, P.R., BROWN, D.A. & CONSTANTI, A. (1982). M-currents and other potassium currents in bullfrog sympathetic neurones. J. Physiol. 330:537-572.

ADAMS, P.R., BROWN, D.A. & JONES, S.W. (1983). Substance P inhibits the M-current in bullfrog sympathetic neurones. Br. J. Pharmac. 79:330-333.

AGARWAL, R.A. & GREENBERG, M.J. (1969). A comparative study of cardioactive agents from bivalve tissue. Comp. Biochem. Physiol. 31:835-850.

AKAIKE, N., BROWN, A.M., DAHL, G., HIGASHI, H., ISENBERG, G., TSUDA, Y. & YATANI, A. (1983). Voltage-dependent activation of potassium current in Helix aspersa neurones by endogenous cellular calcium. J. Physiol. 334:309-324.

AKAIKE, N., LEE, K.S. & BROWN, A.M. (1978). The calcium current of Helix neurone. J. gen. Physiol. 71:509-531.

ASCHER, P. (1972). Inhibitory and excitatory effects of dopamine on Aplysia neurones. J. Physiol. 225:173-209.

ASCHER, P. & CHESNOY-MARCHAIS, D. (1982). Interactions between three slow potassium responses controlled by three distinct receptors in Aplysia neurones. J. Physiol. 324:67-92.

ASCHER, P. & KEHOE, J.S. (1975). Amine and amino acid receptors in gastropod neurons. In: Handbook of Psychopharmacology (Ed. Iverson, L.L., Iverson, S. & Snyder, S.), Plenum Press, New York. Vol. 4:265-310.

ASCHER, P., MARTY, A. & NIELD, T.O. (1978). Life time and elementary conductance of the channels mediating the

excitatory effects of acetylcholine in Aplysia neurones. J. Physiol. 278:177-206.

BADER, C.R., BERNHEIM, L. & BERTRAND, D. (1985). Sodium-activated potassium currents in cultured avian neurones. Nature 317:540-542.

BARBER, A. (1983). Electrical responses of salivary gland cells of the gastropod mollusk Philine aperta to putative neurotransmitters: 3. Amino acids and peptides. Comp. Biochem. Physiol. 75C:161-166.

BARISH, M.E. & THOMPSON, S.H. (1983). Calcium buffering and slow recovery kinetics of calcium-dependent outward current in molluscan neurones. J. Physiol. 337:201-219.

BARKER, J.L. & GAINER, H. (1974). Peptide regulation of bursting pacemaker activity in a molluscan neurosecretory cell. Science 184:1371-1373.

BARKER, J.L. & SMITH, T.G. (1976). Peptide regulation of neuronal membrane properties. Brain Res. 103:167-170.

BARNES, M. (1984). The effect of 5-HT on single channel currents in an identified neurone of Helix aspersa. In: Electropharmacology of the in vitro synapse.

Compiled by Cottrell, G.A. University of St.Andrews Printing Dept. ISBN 0 9510027 08

BARNES, M., COTTRELL, G.A. & DUNBAR, S,J. (1985). The action of cobalt ions on the response of the C1 neurone of Helix aspersa to 5-hydroxytryptamine. J. Physiol. 365:84P.

BARNES, M., COTTRELL, G.A. & DUNBAR, S.J. (1986). Ionic mechanism of a slow voltage-sensitive response to 5-hydroxytryptamine in Helix neurones. In preparation.

BERRY, M.S. & COTTRELL, G.A. (1979). Ionic basis of different synaptic potentials mediated by an identified dopamine-containing neurone in Planorbis. Proc. R. Soc. Lond. B 203:427-444.

BLOOM, F.E. (1983). The endorphins: A growing family of pharmacologically pertinent peptides. Ann. Rev. Pharmac. Toxicol. 23:151-170.

BOER, H.H., SCHOT, L.P.C., REICHELT, D., BRAND, H. & ter MAAT, A. (1984). Ultrastructural immunocytochemical evidence for peptidergic neurotransmission in the pond snail Lymnaea stagnalis. Cell Tissue Res. 238:197-201.

BOER, H.H., SCHOT, L.P.C., VEENSTRA, J.A. & REICHELT, D. (1980). Immunocytochemical identification of neural elements in the central nervous systems of a snail, some insects, a fish and a mammal with an antiserum to the molluscan cardio-excitatory tetrapeptide FMRFamide. Cell Tissue Res. 213:21-27.

BOKISCH, A.J., OSBORNE, N.N. & WALKER, R.J. (1983). Actions of cholecystokinin on neurones in the central nervous system of the snail, Helix aspersa. Comp. Biochem. Physiol. 75C:171-177.

BOYD, P.J. & WALKER, R.J. (1985). Actions of the molluscan neuropeptide FMRF-amide on neurones in the suboesophageal ganglia of the snail Helix aspersa. Comp. Biochem. Physiol. 81C:379-386.

BROWN, B.E. & STARRATT, A.N. (1975). Isolation of proctolin, a myotropic peptide from Periplaneta americana. J. Insect. Physiol. 21:1879-1881.

BROWN, D.A. (1983). Slow cholinergic excitation - a mechanism for increasing neuronal excitability. Trends Neurosci. 6:302-307.

BROWN, D.A. & ADAMS, P.R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+-current in a vertebrate neurone. Nature 283:673-676.

CARPENTER, D.O., SWANN, J.W. & YAROWSKY, P.J. (1977). Effect of curare on responses to different putative neurotransmitters in Aplysia neurones. J. Neurobiol. 8:119-132.

CHAVKIN, C. & GOLDSTEIN, A. (1981). Specific receptor for the opioid peptide dynorphin: Structure activity relations. Proc. Natl. Acad. Sci. USA. 78:6543-6547.

CHIU, A.Y., HUNKAPILLAR, M.W., HELLER, E., STUART, D.K., HOOD, L.E. & STRUMWASSER, F. (1979). Neuropeptide egg- laying hormone of Aplysia: Purification and primary structure. Proc. Natl. Acad. Sci. USA. 76:6656-6660.

COLOMBAIONI, L., PAUPARDIN-TRITSCH, D., VIDAL, P.P. & GERSCHENFELD, H.M. (1985). The neuropeptide FMRF-amide decreases both the Ca^{2+} conductance and a cyclic 3',5'-adenosine monophosphate-dependent K^{+} conductance in identified molluscan neurones. J. Neurosci. 5:2533-2539.

COTTRELL, G.A. (1978). Actions of a "molluscan cardio-excitatory neuropeptide" on identified 5-hydroxytryptamine- containing neurones and their follower neurones in Helix pomatia. J. Physiol.

284:130-131P.

COTTRELL, G.A. (1979). Voltage-dependent action of the molluscan neuropeptide FMRFamide on snail neurones. J. Physiol. 300:41P.

COTTRELL, G.A. (1981). An unusual synaptic response mediated by a serotonin neurone. Q.J. exp. Physiol. 66:475-485.

COTTRELL, G.A. (1982a). FMRFamide neuropeptides simultaneously increase and decrease K^+ currents in an identified neurone. Nature 296:87-89.

COTTRELL, G.A. (1982b). Physiological role of a slow, voltage-sensitive, synaptic response mediated by an identified serotonin-containing neurone. Quart. J. exp. Physiol. 67:179-183.

COTTRELL, G.A. (1983). Actions of FMRFamide and related peptides on snail neurones. In: Molluscan neuroendocrinology (Ed. Lever, J. & Boer, H.H.) North-Holland Publishing Company.

COTTRELL, G.A. & DAVIES, N.W. (1985). Different actions of FMRFamide analogues on identified Helix neurones. J. Physiol. 367:66P.

GERSCHENFELD, H.M. (1981). Role of cAMP in a serotonin-evoked slow inward current in snail neurones. Nature 290:783-785.

DETERRE, P., PAUPARDIN-TRITSCH, D., BOCKAERT, J. & GERSCHENFELD, H.M. (1982). cAMP-mediated decrease in K^+ conductance evoked by serotonin and dopamine in the same neurone: A biochemical and single-cell study. Proc. Natl. Acad. Sci. USA. 79:7934-7938.

DINGLEDINE, R. (1983). N-methyl aspartate activates voltage- dependent calcium conductance in rat hippocampal pyramidal cells. J. Physiol. 343:385-405.

DIONNE, V.E. & STEVENS, C.F. (1975). Voltage dependence of agonist effectiveness at the frog neuromuscular junction. J. Physiol. 251:245-270.

DOCKRAY, G.J. (1985). Characterization of FMRF amide-like immunoreactivity in rat spinal cord by region-specific antibodies in radioimmunoassay and HPLC. J. Neurochem. 45:152-158.

DOCKRAY, G.J., REEVE Jr., J.R., SHIVELY, J., GAYTON, R.J. & BARNARD, C.S. (1983). A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. Nature 305:328-330.

DOCKRAY, G.J., VAILLANT, C. & WILLIAMS, R.G. (1981). New vertebrate brain-gut peptide related to a molluscan neuropeptide and an opioid peptide. Nature 293:656-657.

DODD, J. & KELLY, J.S. (1978). Is somatostatin an excitatory transmitter in the hippocampus? Nature 273:674-675.

DUN, N.J. & MINOTA, S. (1981). Effects of substance P on neurones of the inferior mesenteric ganglia of the guinea-pig. J. Physiol. 321:259-271.

DUNLAP, K. & FISCHBACH, G.D. (1981). Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. J. Physiol. 317:519-535.

ERSPAMER, V. & ANASTASI, A. (1962). Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary gland of Eledone. Experientia 18:58-61.

FINKEL, A.S. (1983). A cholinergic chloride conductance in neurones of Helix aspersa. J. Physiol. 344:119-135.

FRONTALI, N., WILLIAMS, L. & WELSH, J.H. (1967). Heart excitatory and inhibitory substances in molluscan ganglia. Comp. Biochem. Physiol. 22:833-841.

GAYTON, R.J. (1982). Mammalian neuronal actions of FMRFamide and the structurally related opioid Met-enkephalin- Arg^6-Phe^7. Nature 298:275-276.

GERSCHENFELD, H.M., & PAUPARDIN-TRITSCH, D. (1974). Ionic mechanisms and receptor properties underlying the responses of molluscan neurones to 5-hydroxytryptamine. J. Physiol. 243:427-456.

GINSBORG, B.L. (1973). Electrical changes in the membrane in junctional transmission. Biochim. Biophys. Acta. 300:289-318.

GINSBORG, B.L. & KADO, R.T. (1975). Voltage-current relationship of a carbachol-induced potassium-ion pathway in Aplysia neurones. J. Physiol. 245:713-725.

GOLDMAN, D.E., (1943). Potential, impedence and rectification in membranes. J. gen. Physiol. 27:37-60.

GOLDSTEIN, A. & JAMES, I.F. (1984). Multiple opioid receptors, criteria for identification and

classification. Trends Pharmac. Sci. 5:503-505.

GORMAN, A.L.F. & HERMANN, A. (1979). Internal effects of divalent cations on potassium permeability in molluscan neurones. J. Physiol. 296:393-410.

GREENBERG, M.J., PAINTER, S.D. & PRICE, D.A. (1981). The amide of the naturally occurring opioid [Met] enkephalin -Arg^6-Phe^7 is a potent analog of the molluscan neuropeptide FMRFamide. Neuropeptides 1:309-317.

GREENBERG, M.J. & PRICE, D.A. (1979). FMRFamide, a cardioexcitatory neuropeptide of molluscs: An agent in search of a mission. Am. Zool. 19:163-174.

GREENBERG, M.J. & PRICE, D.A. (1983). Invertebrate neuropeptides: native and naturalized. Ann. Rev. Physiol. 45:271-288.

GRUOL, D.L. & WEINREICH, D. (1979). Two pharmacologically distinct histamine receptors mediating membrane hyperpolarization on identified neuronees of Aplysia californica. Brain Res. 162:281-301.

HAAS, H.L. & KONNERTH, A. (1983). Histamine and noradrenaline decrease calcium-activated potassium conductance in hippocampal pyramidal cells. Nature

302:432-434.

HAGIWARA, S. & BYERLY, L. (1981). Calcium channel. Ann. Rev. Neurosci. 4:69-125.

HARTUNG, K. (1985). Potentiation of a transient outward current by Na^+ influx in crayfish neurones. Pflugers Arch. 404:41-44.

HAYNES, L.W. (1980). Peptide neuroregulators in invertebrates. Prog. Neurobiol. 15:205-246.

HELLER, E., KACZMARCK, L.K., HUNKAPILLER, M.W., HOOD, L.E. & STRUMWASSER, F. (1980). Purification and primary structure of two neuroactive peptides that cause bag cell after discharge and egg-laying in Aplysia. Proc. Natl. Acad. Sci. USA. 77:2328-2332.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1978). In vitro release of Leu- and Met-enkephalin from the corpus striatum. Nature 271:677-679.

HERMANN, A. & GORMAN, A.L.F. (1981a). Effects of 4-AP on K^+ currents in a molluscan neurone. J. gen. Physiol. 78:63-86.

HERMANN, A. & GORMAN, A.L.F. (1981b). Effects of TEA on K^+ currents in a molluscan neurone. J. gen. Physiol.

78:87-110.

HEYTLER, P.G. & PRICHARD, W.W. (1962). A new class of uncoupling agents- carbonylcyanide phenylhydrazones (CCP). Biochem. Biophys. Res. Comm. 7:272.

HIGGINS, W.J., PRICE, D.A. & GREENBERG, M.J. (1978). FMRFamide increases the adenylate cyclase activity and cyclic AMP level of molluscan heart. Eur. J. Pharmac. 48:425-430.

HODGKIN, A.L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol. 108:37-77.

HOFMEIER, G. & LUX, H.D. (1981). The time courses of intracellular free calcium and related effects after injection of $CaCl_2$ into neurons of the snail, Helix pomatia. Pflugers Arch. 391:242-251.

HOKFELT, T., JOHANSSON, O., LJUNGDAHL, A., LUNDBERG, J.M. & SCHULTZBERG, M. (1980). Peptidergic neurones. Nature 284:515-521.

HOKFELT, T., LUNDBERG, J.M., SCHULTZBERG, M., JOHANSSON, O., SKIRBOLL, L., ANGGARD, A., FREDHOLM, B., HAMBERGER, B., PERNOW, B., REHFELD, J. & GOLDSTEIN, M. (1980). Cellular localization of peptides in

neuronal structures. Proc. R. Soc. Lond. B 210:63-77.

HUGHES, J., KOSTERLITZ, H.W. & SMITH, T.W. (1977). The distribution of methionine-enkephaline and leucine-enkephalin in the brain and peripheral tissue. Br. J. Pharmac. 61:639-647.

IFSHIN, M.S., GAINER, H., & BARKER, J.L. (1975). Peptide factor extracted from molluscan ganglia that modulates bursting pacemaker activity. Nature 254:72-74.

IUPAC-IUB (1972). Commission on biochemical nomenclature. Pure Appl. Chem. 31:641.

IVERSEN, L.L. (1983a). Nonopioid neuropeptides in mammalian CNS. Ann. Rev. Pharmac. Toxicol. 23:1-27.

IVERSEN, L.L. (1983b). Neuropeptides - what next? Trends Neurosci. 6:293-294.

IVERSEN, L.L., IVERSEN, S.D., BLOOM, F., VARGO, T. & GUILLEMIN, R. (1978). Release of enkephalin from rat globus pallidus in vitro. Nature 271:679-681.

IVERSEN, L.L., IVERSEN, S.D., BLOOM,F., DOUGLAS, C., BROWN, M. & VALE, W. (1978). Calcium-dependent

release of somatostatin and neurotensin from rat brain in vitro. Nature 273:161-163.

IVERSEN, L.L., JESSELL, T. & KANAZAWA, I. (1976). Release and metabolism of substance P in rat hypothalamus. Nature 264:81-83.

IVERSEN, L.L., LEE, C.M., GILBERT, R.F., HUNT, S. & EMSON, P.C. (1980). Regulation of neuropeptide release. Proc. R. Soc. Lond. B 210:91-111.

JAMES, I.F., FISCHLI, W. & GOLDSTEIN, A. (1984). Opioid receptor selectivity of dynorphin gene products. J. Pharmac. exp Therap. 228:88-93.

JAN, L.Y. & JAN, Y.N. (1982). Peptidergic transmission in sympathetic ganglia of the frog. J. Physiol. 327:219-246.

JAN, Y.N. & JAN, L.Y. (1983). A LHRH-like peptidergic neurotransmitter capable of 'action at a distance' in autonomic ganglia. Trends. Neurosci. 6:320-325.

JAN, L.Y., JAN, Y.N. & BROWNFIELD, M.S. (1980). Peptidergic transmitters in synaptic boutons of sympathetic ganglia. Nature 288:380-382.

JAN, Y.N., JAN, L.Y. & KUFFLER, S.W. (1979). A peptide as

a possible transmitter in sympathetic ganglia of the frog. Proc. Natl. Acad. Sci. USA. 76:1501-1505.

KATAYAMA, Y. & NISHI, S. (1982). Voltage-clamp analysis of peptidergic slow depolarizations in bullfrog sympathetic ganglion cells. J. Physiol. 333:305-315.

KATAYAMA, Y., NORTH, R.A. & WILLIAMS, J.T. (1979). The action of substance P on neurones of the myenteric plexus of the guinea-pig small intestine. Proc. R. Soc. Lond. B 206:191-208.

KATZ, B. & MILEDI, R. (1967). A study of synaptic transmission in the absence of nerve impulses. J. Physiol. 192:407-436.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. J. Physiol. 138:63-80.

KEHOE, J. (1972a). Ionic mechanisms of a two-component cholinergic inhibition in Aplysia neurones. J. Physiol. 225:85-114.

KEHOE, J. (1972b). Three ACh receptors in Aplysia neurones. J. Physiol. 225:115-146.

KERKUT, G.A., LAMBERT, J.D.C., GAYTON, R.S., LOKER, J.E. & WALKER, R.J. (1975). Maping of nerve cells in the suboesophageal ganglia of Helix aspersa. Comp. Biochem. Physiol. 50A:1-25.

KLEIN, M., CAMARDO, J. & KANDEL, E.R. (1982). Serotonin modulates a specific potassium current in the sensory neurones that show presynaptic facilitation in Aplysia californica. Proc. Natl. Acad. Sci. USA. 79:5713-5717.

KLEIN, M. & KANDEL, E.R. (1980). Mechanism of calcium current modulation underlying presynaptic facilitation and behavioural sensitization in Aplysia. Proc. Natl. Acad. Sci. USA. 77:6912-6916.

KOSTERLITZ, H.W. (1985). Opioid peptides and their receptors. Proc. R. Soc. Lond. B225:27-40.

LEE, K.S., AKAIKE, N. & BROWN, A.M. (1980). The suction pipette method for internal perfusion and voltage clamp of small excitable cells. J. Neurosci. Methods 2:51-78.

LEHMAN, H.K., PRICE, D.A. & GREENBERG, M.J. (1984). The FMRFamide-like neuropeptide of Aplysia is FMRFamide. Biol. Bull. 167:460-466.

LEVITAN, I.B., HARMAR, A.J. & ADAMS, W.B. (1979). Synaptic and hormonal modulation of a neuronal oscillator: a search for molecular mechanisms. J. exp. Biol. 81:131-151.

LEVITAN, H. & TAUC, L. (1972). Acetylcholine receptors: topographic distribution and pharmacological properties of two receptor types on a single molluscan neurone. J. Physiol. 222:537-558.

LEVITAN, I.B. & TREISTMAN, S.N. (1977). Modulation of electrical activity and cyclic nucleotide metabolism in molluscan nervous system by a peptide containing nervous system extract. Brain Res. 136:307-317.

LLINAS, R., SUGIMORI, M. & SIMON, S.M. (1982). Transmission by presynaptic spike-like depolarization in the squid (Loligo paeli). Proc. Natl. Acad. Sci. USA. 79:2415-2419.

LLOYD, P.E. (1978). Distribution and molecular characteristics of cardioactive peptides in the snail, Helix aspersa. J. Comp. Physiol. 128:269-276.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1977). Endogenous opioid peptides: Multiple agonists and receptors. Nature 267:495-499.

LUNDBERG, J.M. & HOKFELT, T. (1983). Coexistence of peptides and classical neurotransmitters. Trends Neurosci. 6:325-333.

LUX, H.D. & HOFMEIER, G. (1982). Properties of a calcium-activated potassium current in Helix pomatia neurones. Pflugers Arch. 394:61-69.

MAGELBY, K.L. & STEVENS, C.F. (1972). A quantitative description of end-plate currents. J. Physiol. 223:173-197.

MARCHAIS, D. & MARTY, A. (1979). Interaction of permeant ions with channels activated by acetylcholine in Aplysia neurones. J. Physiol. 297:9-45.

MARCHAIS, D. & MARTY, A. (1980). Action of glucoseamine on acetylcholine sensitive channels. J. Membrane Biol. 56:43-48.

MARTY, A. & ASCHER, P. (1978). Slow relaxations of acetylcholine-induced potassium currents in Aplysia neurones. Nature 274:494-497.

MARUYAMA, Y. & PETERSEN, O.H. (1982). Cholecystokinin activation of single-channel currents is mediated by internal messenger in pancreatic acinar cells. Nature 300:61-63.

MAYERI, E., BROWNWELL, P. & BRANTON, W.D. (1979). Multiple, prolonged actions of neuroendocrine bag cells on neurones in Aplysia. II Effects on beating pacemaker and silent neurones. J. Neurophysiol. 42:1185-1197.

MAYERI, E., BROWNWELL, P., BRANTON, W.D. & SIMON, S.B. (1979). Multiple, prolonged actions of neuroendocrine bag cells on neurones in Aplysia. I Effects on bursting pacemaker neurones. J. Neurophysiol. 42:1165-1184.

McCARTHY, P.W. & COTTRELL, G.A. (1984). Responses of mouse spinal neurones in culture to locally applied Phe-Met-Arg-Phe-NH_2. Comp. Biochem. Physiol. 79C:383-387.

MEECH, R.W. (1974). The sensitivity of Helix aspersa neurones to injected Ca^{2+} ions. J. Physiol. 237:259-277.

MEECH, R.W. & STANDEN, N.B. (1975). Potassium activation in Helix aspersa neurones under voltage clamp: A component mediated by calcium influx. J. Physiol. 249:211-239.

MEECH, R.W. & THOMAS, R.C. (1977). The effect of calcium injection on the intracellular sodium and pH of snail

neurones. J. Physiol. 265:867-879.

MEECH, R.W. & THOMAS, R.C. (1980). Effect of calcium chloride injections on the membrane potential and internal pH of snail neurones. J. Physiol. 298:111-129.

MUDGE, A.W., LEEMAN, S.E. & FISCHBACH, G.D. (1979). Enkephalin inhibits release of substance P from sensory neurones in culture and decreases action potential duration. Proc. Natl. Acad. Sci. USA. 76:526-530.

MUNEOKA, Y. & MATSUURA, M. (1985). Effects of the molluscan neuropeptide FMRFamide and the related opioid peptide YGGFMRFamide on Mytilus muscle. Comp. Biochem. Physiol. 81C:61-70.

MURASE, K. & RANDIC, M. (1984). Actions of substance P on rat spinal dorsal horn neurones. J. Physiol. 346:203-217.

NAGLE, G.T. (1981). The molluscan cardioactive neuropeptide FMRFamide: Subcellular localization in bivalve ganglia. J. Neurobiol. 12:599-611.

NAGLE, G.T. (1982). The molluscan neuropeptide FMRFamide: calcium-dependent release and blood levels in

Macrocallista (Bivalvia). Life Sci. 30:803-807.

NEHER, E. & SAKMANN, B. (1975). Voltage-dependence of drug-induced conductance in frog neuromuscular junction. Proc. Natl. Acad. Sci. USA. 72:2140-2144.

NICOLL, R.A. (1978). The action of thyrotropin-releasing hormone, substance P and related peptides on frog spinal motoneurones. J. Pharmac. exp. Ther. 207:817-824.

NORTH, R.A. & WILLIAMS, J.T. (1985). On the potassium conductance increase by opioids in rat locus coeruleus neurones. J. Physiol. 364:265-280.

ONSAGER, L. (1934). Deviations from Ohms law in weak electrolytes. J. chem. Phys. 2:599-615.

O'SHEA, M. & BISHOP, C.A. (1982). Neuropeptide proctolin associated with an identified skeletal motoneurone. J. Neurosci. 2:1242-1251.

O'SHEA, M. & SCHAFFER, M. (1985). Neuropeptide function: The invertebrate contribution. Ann. Rev. Neurosci. 8:171-198.

OTSUKA, M. & KONISHI, S. (1976). Release of substance

P-like immunoreactivity from isolated spinal cord of newborn rat. Nature 264:83-84.

OTSUKA, M. & KONISHI, S. (1983). Substance P - the first peptide neurotransmitter? Trends Neurosci. 6:317-320.

OTSUKA, M., KONISHI, S., YANAGISAWA, M., TSUNOO, A. & AKAGI, H. (1982). Role of substance P as a sensory transmitter in spinal cord and sympathetic ganglia. In: Substance P in the nervous system. Pitman, London (Ciba Foundation symposium 91) p 13-34.

PAINTER, S.D., MORLEY, J.S. & PRICE, D.A. (1982). Structure-activity relations of the molluscan neuropeptide FMRFamide on some molluscan muscles. Life Sci. 31:2471-2478.

PELLMAR, T.C. (1981). Does cyclic 3',5'-adenosine monophosphate act as second messenger in a voltage-dependent response to 5-hydroxytryptamine? Br. J. Pharmac. 74:747-756.

PELLMAR, T.C. (1984). Enhancement of inward current by serotonin in Aplysia. J. Neurobiol. 15:13-25.

PELLMAR, T.C. & CARPENTER, D.O. (1980). Serotonin induces a voltage-sensitive calcium current in neurones of

Aplysia californica. J. Neurophysiol. 44:423-439.

PRICE, D.A., COTTRELL, G.A., DOBLE, K.E., GREENBERG, M.J. JORENBY, W., LEHMAN, H.K. & RIEHM, J.P. (1985). A novel FMRFamide-related peptide in Helix: pQDPFLRFamide. Biol. Bull. 169:256-266.

PRICE, D.A. & GREENBERG, M.J. (1977). Structure of a molluscan cardioexcitatory neuropeptide. Science 197:670-671.

PRICE, D.A. & GREENBERG, M.J. (1980). Pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. Gen. Pharmac. 11:237-241.

PURVES, R.D. (1977). The time course of cellular responses to iontophoretically applied drugs. J. theor. Biol. 65:327-344.

REHFELD, J.F. (1985). Neuronal cholecystokinin: One or multiple transmitters? J. Neurochem. 44:1-10.

RENAUD, L.P. & PADJEN, A. (1978). Electrophysiological analysis of peptide actions in neural tissue. In: Centrally acting peptides. (Ed. Hughes, J., Macmillan Press Ltd.).

ROSE, B. & LOEWENSTEIN, W.R. (1975). Calcium ion

distribution in cytoplasm visualised by aequorin: diffusion in cytosol restricted by energised seqestering. Science 190:1204-1206.

ROTHMAN, B.S., MAYERI, E., BROWN, R.O., YUAN, P.-M. & SHIVELY, J.E. (1983). Primary structure and neuronal effects of -bag cell peptide, a second candidate neurotransmitter encoded by a single gene in bag cell neurones of Aplysia. Proc. Natl. Acad. Sci. USA. 80:5753-5757.

SAKAI, M., SWARTZ, B.E. & WOODY, C.D. (1979). Controlled micro release of pharmacological agants: measurements of volume ejected in vitro through fine tipped glass microelectrodes by pressure. Neuropharmac. 18:209-213.

SCHELLER, R.H., ROTHMAN, B.S. & MAYERI, E. (1983) A single gene encodes multiple peptide-transmitter candidates involved in a stereotyped behaviour. Trends Neurosci. 6:340-345.

SCHOT, L.P.C. & BOER, H.H. (1982). Immunocytochemical demonstration of peptidergic cells in the pond snail Lymnaea stagnalis with an antiserum to the molluscan cardioactive tetrapeptide FMRF-amide. Cell Tissue Res. 225:347-354.

SCHULZ, R., WUSTER, M. & HERZ, A. (1981). Pharmacological characterization of the ϵ-opiate receptor. J. Pharmac. exp. Ther. 216:604-606.

SHUSTER, M.J., CAMARDO, J.S., SIEGELBAUM, S.A. & KANDEL, E.R. (1985). Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K^+ channel of Aplysia sensory neurones in cell-free membrane patches. Nature 312:392-395.

SIBLEY, D.R. & LEFKOWITZ, R.J. (1985). Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 317:124-129.

SIEGLEBAUM, S.A., CAMARDO, J.S. & KANDEL, E.R. (1982). Serotonin and cyclic AMP close single K^+ channels in Aplysia sensory neurones. Nature 229:413-417.

SNYDER, S.H. (1980). Brain peptides as neurotransmitters. Science 209:976-983.

STEFANO, G.B., S.-ROZSA, K. & HIRIPI, L. (1980). Actions of methionine enkephaline and morphine on single neuronal activity in Helix pomatia. Comp. Biochem. Physiol. 66C:193-198.

SWAN, J.W. & CARPENTER, D.O. (1975). Organization of

receptors for neurotransmitters on Aplysia neurones. Nature 258:751-754.

TANG, J., YANG, H.Y.T. & COSTA, E. (1984). Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH_2-like immuno- reactivity. Proc. Natl. Acad. Sci. USA. 81:5002-5005.

THOMPSON, S.H. (1977). Three pharmacologically distinct K^+ channels in molluscan neurones. J. Physiol. 265:465-488.

TILLOTSON, D. & HORN, R. (1978). Inactivation without facilitation of calcium conductance in caesium loaded neurones of Aplysia. Nature 273:312-314.

VINCENT, J.D. & BARKER, J.L. (1979). Substance P: Evidence for diverse roles in neuronal function from cultured mouse spinal neurones. Science 205:1409-1411.

WEBER, E., EVANS, C.J. & BARCHAS, J.D. (1983). Multiple endogenous ligands for opioid receptors. Trends Neurosci. 6:333-336.

WEBER, E., EVANS, C.J., SAMUELSSON, S. & BARCHAS, J.D. (1981). Novel peptide neuronal system in rat brain and pituitary. Science 214:1248-1251.

WERZ, M.A. & MACDONALD, R.L. (1982). Heterogeneous sensitivity of cultured dorsal root ganglion neurones to opioid peptides selective for μ- and δ-opioid receptors. Nature 299:730-733.

WERZ, M.A. & MACDONALD, R.L. (1983). Opioid peptides with differential affinity for mu and delta receptors decrease sensory neuron calcium-dependent action potentials. J. Pharmac. exp. Ther. 227:394-402.

WERZ, M.A. & MACDONALD, R.L. (1984). Dynorphin reduces voltage- dependent calcium conductance of mouse dorsal root ganglion neurones. Neuropeptides 5:253-256.

WILSON, W.A. & GOLDNER, M.M. (1975). Voltage clamping with a single microelectrode. J. Neurobiol. 6:411-422.

WITTE, O.W., SPECKMANN, E.J. & WALDEN, J. (1985). Acetylcholine responses of identified neurones in Helix pomatia -III Ionic composition of the depolarizing currents induced by acetylcholine. Comp. Biochem. Physiol. 80C:37-45.